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Role of the protein tyrosine phosphatase DEP-1 in Src activation and the mediation of biological cell functions of endothelial and breast cancer cells

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Cette thèse intitulée:

Role of the protein tyrosine phosphatase DEP-1 in Src activation and the mediation of biological cell functions in endothelial and breast cancer cells

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Résumé

L'implication des protéines tyrosines phosphatases (PTPs) dans la régulation de la signalisation et la médiation des fonctions cellulaires a été bien établie dans les dernières années. Cependant, les mécanismes moléculaires par lesquels les PTPs régulent les processus fondamentaux tels que l'angiogenèse demeurent méconnus. Il a été rapporté que l'expression de la PTP DEP-1 (Density-enhanced phosphatase 1) augmente avec la densité cellulaire et corrèle avec la déphosphorylation du récepteur VEGFR2. Cette déphosphorylation contribue à l'inhibition de contact dans les cellules endothéliales à confluence et diminue l'activité du VEGFR2 en déphosphorylant spécifiquement ses résidus catalytiques Y1054/1059. De plus, la plupart des voies de signalisation en aval du VEGFR2 sont diminuées sauf la voie Src-Gab1-AKT. DEP-1 déphosphoryle la Y529 de Src et contribue à la promotion de la survie dans les cellules endothéliales.

L'objectif de cette thèse est de mieux définir le rôle de DEP-1 dans la régulation de l'activité de Src et les réponses biologiques dans les cellules endothéliales. Nous avons identifié les résidus Y1311 et Y1320 dans la queue C-terminale de DEP-1 comme sites majeurs de phosphorylation en réponse au VEGF. La phosphorylation de ces résidus est requise pour l'activation de Src et médie le remodelage des jonctions cellules-cellules dépendantes de Src. Ce remodelage induit la perméabilité, l'invasion et la formation de capillaires en réponse au VEGF. Nos résultats démontrent que la phosphorylation de DEP-1 sur résidu tyrosine est requise pour diriger la spécificité de DEP-1 vers son substrat Src. Les travaux révèlent pour la première fois un rôle positif de DEP-1 sur l'induction du programme angiogénique des cellules endothéliales.

En plus de la phosphorylation sur tyrosine, DEP-1 est constitutivement phosphorylé sur la thréonine 1318 situé à proximité de la Y1320 en C-terminal. Cette

localisation de la T1318 suggère que ce résidu pourrait être impliqué dans la régulation de la Y1320. En effet, nous avons observé que la T1318 de DEP-1 est phosphorylée potentiellement par CK2, et que cette phosphorylation régule la phosphorylation de DEP-1 sur tyrosine et sa capacité de lier et d'activer Src. En accord avec ces résultats, nos travaux révèlent que la surexpression du mutant DEP-1 T1318A diminue le remodelage des jonctions cellules-cellules et par conséquent la perméabilité. Nos résultats suggèrent donc que la T1318 de DEP-1 constitue un nouveau mécanisme de contrôle de la phosphorylation sur tyrosine et que ceci résulte en l'activation de Src et l'induction des fonctions biologiques des cellules endothéliales en réponse au VEGF.

Suite à ces travaux dans les cellules endothéliales qui démontrent un rôle positif de DEP-1 dans la médiation des réponses angiogéniques, nous avons voulu approfondir nos connaissances sur l'implication potentielle de DEP-1 dans les cellules cancéreuses où l'activité de Src est requise pour la progression tumorale. Malgré le rôle connu de DEP-1 comme suppresseur tumoral dans différents types de cancer, nous avons émis l'hypothèse que DEP-1 pourrait promouvoir les fonctions biologiques dépendantes de Src telles que la migration et l'invasion dans les cellules cancéreuses. Ainsi, nous avons observé que l'expression de DEP-1 est plus élevée dans les lignées basales de cancer du sein qui sont plus invasives comparativement aux lignées luminales peu invasives. Dans les lignées basales, DEP-1 active Src, médie la motilité cellulaire dépendante de Src et régule la localisation des protéines impliquées dans l'organisation du cytosquelette. L'analyse d'un micro-étalage de tissu a révélé que l'expression de DEP-1 est associée avec une réduction tendencielle de survie des patients. Nos résultats proposent donc, un rôle de promoteur tumoral pour DEP-1 dans la progression du cancer du sein.

Les travaux présentés dans cette thèse démontrent pour la première fois que DEP-1 peut agir comme promoteur des réponses angiogéniques et du phénotype pro-invasif des lignées basales du cancer du sein probablement du à sa capacité d'activer Src. Nos résultats suggèrent ainsi que l'expression de DEP-1 pourrait contribuer à la

progression tumorale et la formation de métastases. Ces découvertes laissent donc entrevoir que DEP-1 représente une nouvelle cible thérapeutique potentielle pour contrer l'angiogenèse et le développement du cancer.

Mots-clés : Angiogenèse, cellules endothéliales, DEP-1, VEGFR2, Src, VE-cadhérine, perméabilité, invasion, cancer du sein

Summary

The implication of protein tyrosine phosphatases (PTPs) in the regulation of cell signalling events and the mediation of cellular functions in response to growth factors such as VEGF has been well-established in the last years. Nonetheless, molecular mechanisms by which PTPs regulate fundamental processes such as angiogenesis are not well-characterized. Expression of the PTP DEP-1 (Density-enhanced phosphatase 1) was reported to increase with cell density and was associated with VEGFR2 dephosphorylation contributing to cell contact inhibition in confluent endothelial cells. We previously demonstrated that DEP-1 attenuates VEGFR2 activity by dephosphorylation of its Y1054/1059 leading to decreased activation of major signalling pathways downstream of VEGFR2 with exception of the Src-Gab1-AKT pathway. Increasing Src activity due to DEP-1-mediated dephosphorylation of its Y529 promotes endothelial cell survival.

The objective of this thesis was to gain a better understanding of the role of DEP-1 in the regulation of the Src activity and of biological responses in endothelial cells. We identified tyrosine Y1311 and Y1320 in the C-terminal tail of DEP-1 as major phosphorylation sites in response to VEGF. These residues are required for Src activation and mediate the Src-dependent remodelling of endothelial cell junctions inducing permeability, invasion and capillary formation upon VEGF stimulation. We showed that VEGF-induced DEP-1 tyrosine phosphorylation directs DEP-1 specificity towards its substrate Src. Our results thus highlighted for the first time the promoting role of DEP-1 on the angiogenic program in endothelial cells.

In addition to tyrosine phosphorylation, DEP-1 is constitutively phosphorylated on a threonine residue (T1318) proximal to Y1320 in its C-terminal tail suggesting it might be involved in the regulation of Y1320. Indeed, we found that DEP-1 T1318 is phosphorylated, potentially by CK2, and regulates the tyrosine

phosphorylation of DEP-1 and its ability to bind to and activate Src. Consistent with this, remodelling of endothelial cell junctions and permeability are impaired in endothelial cells expressing the DEP-1 T1318 mutant. Thus, DEP-1 phosphorylation on T1318 displays a regulatory control over DEP-1 tyrosine phosphorylation and subsequently Src activation and endothelial cell functions in response to VEGF.

Our results demonstrating that DEP-1 promotes angiogenic cell responses in endothelial cells, prompted us to consider a possible involvement of DEP-1 in cancer cells, where Src activation has been linked to cancer progression. Thus, although, DEP-1 is believed to act as a tumour suppressor in different cancer types, we hypothesized that it might also promote Src-dependent functions such as migration and invasion in cancer cells. Interestingly, we found that DEP-1 is higher expressed in more invasive basal-like breast cancer cells than in luminal-like cell lines. Moreover, DEP-1 is implicated in the regulation of Src activity, Src-mediated cell motility and appropriate localization of proteins mediating cytoskeletal organization in basal-like breast cancer cell lines. To further support these results, analysis of a breast cancer tissue microarray revealed that DEP-1 expression is associated with a tendency towards reduced overall survival. Thus, our results provide first evidence for a tumour-promoting role of DEP-1 in breast cancer.

Altogether, the work performed in the context of this thesis revealed that DEP-1 can similarly behave as a promoter of the angiogenic response and of the pro-invasive phenotype in basal-like breast cancer cell lines, most likely due to its ability to activate Src. This suggests for the first time that DEP-1 expression could contribute to tumour progression and the formation of metastases, and as such, represent a potential new target for anti-angiogenic and anti-cancer therapy.

Keywords : Angiogenesis, endothelial cells, DEP-1, VEGFR2, Src, VE-cadherin, permeability, invasion, breast cancer

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List of abbreviations

BAD	Bcl-2 associated death promoter
Cdc42	Cell division control protein 42
CHO	Chinese hamster ovary
Csh	Csk-homology kinase
Csk	C-terminal Src kinase
DEP-1	Density enhanced phosphatase-1
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial NO-synthase
EMT	Epithelial to mesenchymal transition
ERK1/2	Extracellular regulated kinase 1/2
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
Gab1	Grb2-associated-binding protein 1
GLEPP1	Glomerular epithelial protein 1
Grb2	Growth factor receptor bound protein 2
Her2/ErbB2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
HIF1	Hypoxia induced factor 1
HUVEC	Human umbilical vein endothelial cells
JAK	Janus kinase
kDa	kiloDalton
KDR	Kinase insert domain receptor

LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
PAG	Phosphoprotein asscociated with glycosphingo-lipid-enriched microdomains
PAK	p21 activated kinase
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PIGF	Placental growth factor
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
pNPP	Para-nitrophenyl phospahte
PTP	Protein Tyrosine Phosphatase
Rac	Ras-related C3 botulinum toxin substrate
Ras	Rat sarcoma
RPTP	Receptor-like protein tyrosine phosphatase
RTK	Receptor tyrosine kinase
ROS	Reactive oxygen species
SAP-1	Stomach cancer-associated protein tyrosine phosphatase
SFK	Src family of kinases
SH	Src-homology domain
SHB	SH2 domain containing adaptor protein B
SHP-1	Src-homology 1 domain-containing protein tyrosine phosphatase
SHP-2	Src-homology 2 domain-containing protein tyrosine phosphatase
SNP	Single nucleotide polymorphisms
Src	Sarcoma

SSTR1	Somatostatin Receptor 1
STAT	Signal transducers and activators of transcription
sVEGFR1	soluble VEGF receptor 1
sVEGFR2	soluble VEGF receptor 2
TCR	T cell receptor
TC-PTP	T cell – protein tyrosine phosphatase
TGF-β1	Transforming growth factor β1
TPC	Tumour propagating cell
TSP-1	Thrombospondin-1
VE-Cadherin	Vascular endothelial-cadherin
VEGF	Vascular endothelial growth factor
VEGFR1/Flt-1	Vascular endothelial growth factor receptor 1
VEGFR2/Flk-1/KDR	Vascular endothelial growth factor receptor 2
VEGFR3/Flt-4	Vascular endothelial growth factor receptor 3
VE-PTP	Vascular endothelial protein tyrosine phosphatase
VPF	Vascular Permeability Factor
ZO-1	Zona occludin-1

Für meine Lieben

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CHAPTER I

Introduction

Angiogenesis is the formation of new blood vessels starting from already existing vessels and is essential for life and development. Hence, blood vessels are formed early to provide oxygen and nutrients to developing organs. In adults, angiogenesis occurs only in particular physiological situations including the ovarian cycle in women, wound healing processes, and placenta formation in pregnancy. However, angiogenesis can also contribute to the development of pathological conditions when it is either over- or under-regulated, for instance, in atherosclerosis, arthritis, cardiac ischemia, diabetic retinopathy, and during cancer progression. Understanding the complex regulation of vessel formation during physiological but especially in pathological situations is therefore of significant interest in medical research. Most notably, in cancer, angiogenesis is implicated in the development and the progression and the malignancy of the disease. Cancer-related angiogenesis is responsible for various steps of cancer progression including rapid tumour growth and metastasis formation. To date, several anti-angiogenic therapies targeting the VEGF pathway have been developed and have been shown to successfully inhibit local tumour growth and metastasis formation. However, anti-angiogenic therapies were less beneficial for cancer patients than predicted (1, 2). Even worse, it was suggested that potential adverse effects of anti-angiogenic therapies might select for tumour cells with higher fitness and higher invasive capacities (3, 4). In agreement with this hypothesis, it was recently demonstrated in a mouse model that cancer cell invasion as well as metastasis formation were increased during “short term” anti-angiogenic treatment (5-7). Thus, the ongoing challenge in the application of anti-angiogenic therapies in cancer is to overcome the tumour evasion of these therapies. Therefore, a deeper understanding of the various components of vessel formation and resistance to

anti-angiogenic treatment is required to design novel and more efficient anti-angiogenic therapeutic approaches.

1.1 Capillary formation under normal conditions

Blood vessels are the first functional organ system formed in the embryo. Initial evidence of a primitive vascular network occurs at day 8.0 of gestation in the mouse embryo (8). The formation of new vessels during development involves two processes. The early simple circulatory loop of the vascular system is formed by vasculogenesis and implicates the differentiation of progenitors of endothelial cells, called angioblasts, followed by their assembly into a simple labyrinth of small capillaries. Although tubes adopt a branched pattern, they appear diffuse and not well organized. During the angiogenic phase, the simple vascular plexus expands due to endothelial cell proliferation and remodels into a more highly organized vascular network (figure 1). This matured vascular system is characterized by blood vessels that are ramified into smaller capillaries, increasing the network complexity. The maturation process involves the recruitment of pericytes and vascular smooth muscle cells (VSMCs) that promote vessel stabilization and quiescence (9).

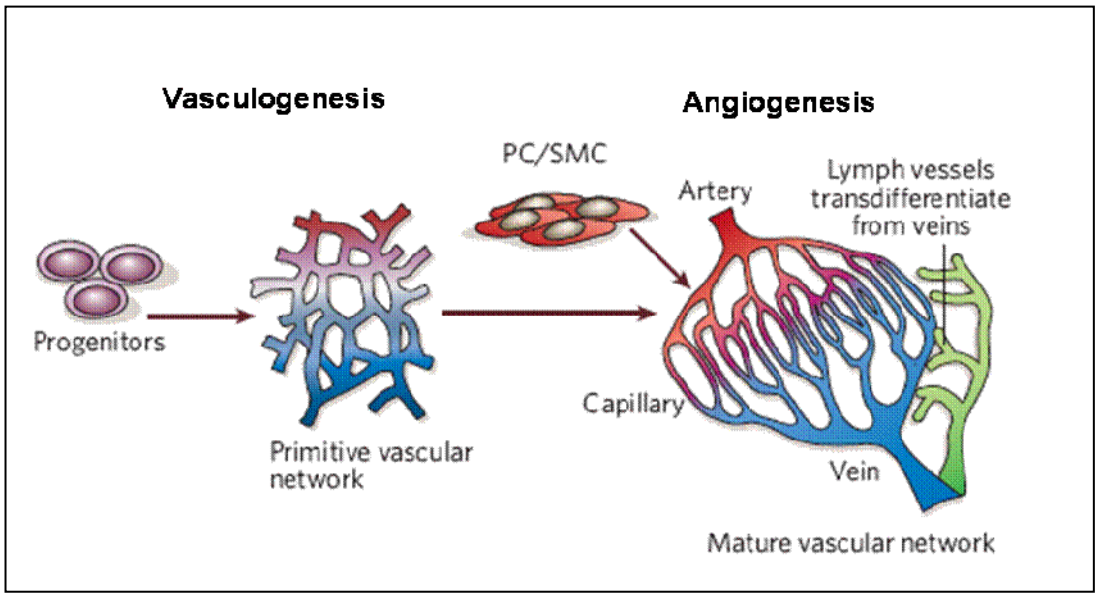

Figure 1 : Development of the vascular system.

During development a primitive vascular network is formed by angioblasts, the endothelial cell progenitors. This first vascular network is formed through vasculogenesis. The tubular network develops and becomes more complex during angiogenesis. Pericytes (PC) and smooth muscle cells (SMC) are recruited to the tubular network to stabilize the major and highly organized vascular system. Adapted from (9).

Vascular sprouting is an early step during the angiogenic process that initiates the formation of new vessels. This process encompasses the activation of quiescent endothelial cells by angiogenic stimuli and results in small sprouts. Subsequently, endothelial cells loosen their cell-cell junctions and begin to degrade the local basal membrane. Only a fraction of endothelial cells adopt the specialized tip cell phenotype, generating an endothelial cell that can sense angiogenic factors (figure 2A). Tip cells distinguish themselves by a high number of filopodia as well as high expression of VEGFR2 and the Notch ligand DII4, which both favour the tip cell phenotype. Tip cells direct the migration of the sprout to the stimulus (10-12). Tip cells induce a lateral inhibition mechanism on neighbouring endothelial cells, which is mediated by the DII4/Notch pathway. As a consequence, VEGF receptors are

downregulated and the motile, invasive tip cell phenotype is suppressed in these neighbouring cells (13, 14). They stay behind the tip cells and form the stalk of the sprout, maintaining vessel integrity and perfusion of the new vessel (figure 2B). Stalk cells undergo cell proliferation to elongate the new vessel (15, 16). Upon contact with other vessels, the motile tip cell phenotype is suppressed in these cells and they convert into stalk cells. The quiescent status of the newly formed vessel is established by the tightening of cell-cell junctions and the recruitment of pericytes and VSMCs as well as deposition of the extracellular matrix (ECM) (17). The equilibrium and the fine-tuning of different angiogenic factors during vessel formation are essential for proper angiogenesis.

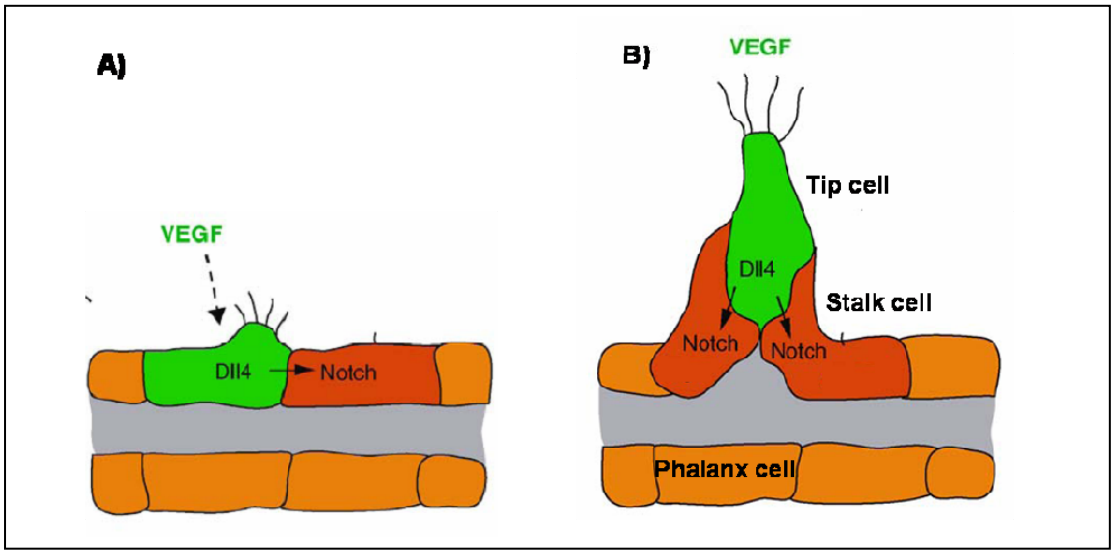

Figure 2: Endothelial cell dynamics during vascular sprouting.

A) Following VEGF stimulation, endothelial cells form protrusions and adopt a motile phenotype along with the loosening of cell-cell junctions. Tip cells are selected by Notch and DII4 signalling at the angiogenic front. VEGF increases the expression levels of DII4 in tip cells, resulting in elevated Notch levels in adjacent cells and the subsequent inhibition of VEGF receptor expression in these cells. B) Tip cells have many filopodia to sense their environment and to direct the sprout towards the angiogenic stimuli. Adjacent cells form the stalk of the sprout, and elongate the new vessel. Quiescent phalanx cells maintain vessel integrity and perfusion. Adapted from (18).

1.2 Regulation of angiogenesis – a balance between pro- and anti-angiogenic factors

Angiogenesis is regulated by several pro- and anti-angiogenic factors to maintain the balance for appropriate formation of new vessels. A main pro-angiogenic factor is VEGF. In 1983, Senger and colleagues identified a factor in the conditioned medium of a tumour cell line able to induce vascular leakage in the skin, which they called VPF (vascular permeability factor). They proposed that this factor can induce high permeability in tumour blood vessels (19). In 1989, Ferrara and colleagues isolated a diffusible endothelial-cell specific mitogen which they called VEGF (20). In the same year, cDNA cloning revealed that both factors are the same molecule (21, 22). Gene knockout studies revealed that the knockout of both VEGF gene copies is lethal at embryonic day 8-9 and the inactivation of one single allele is sufficient to induce severe angiogenic failure and embryonic lethality between day 10 and 12 (23, 24). The VEGF family contains different genes, VEGF-A, -B, -C, VEGF-D and PIGF. In addition, there are structurally related forms of VEGF, namely, VEGF-E, found in parapoxvirus, and VEGF in snake venoms, which are generally known as VEGF-F (25, 26). Alternative exon splicing of the VEGF-A gene results in four distinct isoforms, which are VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206}. (27, 28) The most frequent isoform is VEGF-A_{165}. This isoform was the first described and is

a potent inducer of endothelial permeability (29, 30). Other VEGF isoforms including VEGF-$A_{111,}$ VEGF-A_{145}, VEGF-A_{162} and VEGF-A_{183} are less frequent (31, 32). In contrast to mouse models only expressing the less frequent VEGF-A_{121} or VEGF-A_{189} isoform, mice exclusively expressing VEGF-A_{165} are perfectly viable and healthy. Moreover, it was shown that VEGF-A_{165}, in contrast to VEGF-A_{121} or VEGF-A_{189}, could completely rescue tumour growth in $VEGF^{-/-}$ tumorigenic mice (33-35). These studies support the principal role of VEGF-A_{165} as an effector of VEGF functions. The VEGF-A isoforms have different capacities to bind to heparan sulphate proteoglycan (HSPG). Proteolytic cleavage of the VEGF-A isoforms abrogates their binding to heparin and consequently generates free soluble fragments, regulating the local VEGF activity and its bioavailability. While VEGF-A_{189} and VEGF-A_{206} are completely sequestered in the ECM due to their high affinity to bind to HSPG, VEGF-A_{121} fails to bind to heparin and is freely diffusible (29). Interestingly, the HSPG-bound form of VEGF-A_{165} is in equilibrium with the soluble form that is generated by proteolytic cleavage (36, 37). Thus, the VEGF-A_{165} isoform combines optimal bioavailability characteristics due to intermediate extracellular matrix binding with high biological potency. VEGF-C and VEGF-D are generated through proteolytic processing and are mainly involved in the regulation of lymphatic angiogenesis (38-40). Several factors can regulate the expression of angiogenic stimulators. For instance, hypoxia-inducible factor (HIF-1) can induce the expression of several VEGF forms and the VEGF receptors in physiological and pathological conditions. Moreover, a wide range of transcription factors and ROS can modulate the expression levels of VEGF (41-43).

Further specific factors acting on the vascular endothelium are four angiopoietin members and members of the ephrin family (44-48). There are also multiple other factors including members of the HGF, FGF, PDGF and TGFβ families that can directly act on endothelial cells or induce VEGF expression in cells (49-51). Even if VEGF is the main angiogenic factor, it has to work in concert with

these factors to ensure proper vessel formation, maturation and stabilisation of the tube network.

Anti-angiogenic factors, also called natural inhibitors of angiogenesis, are TSP-1, angiostatin and endostatin. Endostatin was purified from tumour cell-conditioned medium and is a fragment of collagen type XVIII (52). It inhibits endothelial cell proliferation and migration and induces apoptosis (53). Angiostatin, a fragment of plasminogen, was purified from serum of tumour-bearing mice and inhibits the proliferation of endothelial cells in growing vessels (52, 54). TSP-1, a heparin-binding protein, was purified as the first endogenous angiogenic inhibitor. It is stored in the ECM and inhibits endothelial cell proliferation (55, 56). Angiogenesis in physiological situations has to be tightly regulated. The balance of pro-angiogenic factors as well as endogenous anti-angiogenic factors is crucial for appropriate angiogenesis.

1.3 Angiogenesis in pathological situations – the loss of the balance of angiogenic factors

In pathologies such as cancer, the local balance between pro and anti-angiogenic factors is shifted towards pro-angiogenic factors. This imbalance is often found in the primary tumour, and in late stage cancer it is also found at sites of distant metastasis (57). Angiogenesis is one of the hallmarks of cancer and a key element in cancer progression (58). Once the developing tumour reaches a diameter of few mm, blood and nutrient flow inside the tumour lesion are diminished, initiating hypoxia and nutrient deprivation (59). Hypoxia induces HIF-1α expression, which in turn induces the secretion of stimulatory factors from different cell types including endothelial, stromal and tumour cells (43, 60). These conditions allow the “angiogenic switch” and cancer progression (61). Cancer-associated angiogenesis is significantly altered by many processes, giving rise to vessels that never become quiescent. The tumour

vessel network is characterized by an irregular formation with tortuous and typically fenestrated vessels and without stable cell-cell contacts, thus generating a highly permeable phenotype (30). Tumour cells and a variety of tumour-associated cells including fibroblasts create their microenvironment by the secretion of angiogenic factors and deposition of ECM to influence the angiogenic response in a tumour (62-64). Due to an overwhelming secretion of cytokines and growth factors especially by tumour cells, signalling becomes dysregulated, leading to a constant activation of normal and quiescent EC. The high PDGF concentration secreted by platelets or activated EC in normal angiogenesis stimulates perivascular cells to cover newly formed vessels. In contrast, vessels remain uncovered and consequently unstable and activated in tumour angiogenesis (65, 66). Thus, the overall angiogenic response is poor due to constant vessel growth without maturation and stabilization although PDGF is secreted. Moreover, inflammatory cells accumulate in the tumour lesion, in turn releasing more angiogenic factors (67, 68). These conditions prompted Dvorak to describe these lesions as "wounds that do not heal" due to aberrant vasculature, inflammatory cell accumulation and sites of necrosis (62, 68, 69).

1.4 Transmission of the angiogenic signal through VEGF receptors

The VEGF receptor family is composed of 3 members that are structurally homologous. VEGFR2 (Flk-1, KDR) is essential for the vascular system in development and life, as illustrated by VEGFR2$^{-/-}$ knockout studies. Mice lacking Flk-1 die at embryonic day 8.5 due to failures in the development of angiogenic structures (70). Flk-1 is the main transducer of VEGF-A-induced signals that maintain important biological responses of endothelial cells such as permeability, migration and proliferation. VEGF-A is the main VEGFR2 ligand, but VEGF-C and -D can also bind to it, although with a lesser affinity than to their principal receptor VEGFR3 (71). VEGFR2 is composed of an extracellular domain consisting of

numerous Ig-like domains, followed by a transmembrane domain and an intracellular portion exhibiting tyrosine kinase activity (see figure 3) (72-74). Alternative splicing can generate a soluble form of VEGFR2, called sVEGFR2 (figure3). This isoform is present in various tissues and binds to VEGF-C, but not VEGF-A. Binding of sVERGR2 to VEGF-C blocks lymphatic endothelial cell proliferation, suggesting that sVEGFR2 acts as a competitor for VEGF-C-binding to VEGFR3 (75). Moreover, sVEGFR2 can bind to the full length VEGFR2 molecule, inhibiting tumour angiogenesis (76). In adults, VEGFR2 is prominently expressed in endothelial cells, but also in a range of non-endothelial cells (77).

Interestingly, while VEGFR2 has promoting roles, VEGFR1 (Flt-1) suppresses angiogenesis in early embryogenesis as demonstrated in FLt-$1^{-/-}$ mice, which die at E8.5-9 due to overgrowth and disorganization of blood vessels (78). In contrast, the lack of the intracellular domain of Flt-1 did not affect the viability and blood vessel formation in mice, suggesting that the extracellular and transmembrane domain is sufficient to suppress angiogenesis in early development (79). VEGFR1-dependent signalling is implicated in certain phenomena of tumour angiogenesis, in inflammation-associated angiogenesis and the progression of rheumatoid arthritis and arteriosclerosis (80-84). Interestingly, the mRNA of VEGFR1 also codes for a shorter protein that gives rise to a soluble form of VEGFR1 (sVEGFR1), consisting only of the extracellular domain (figure 3) (85). The soluble form of VEGFR1 is abnormally expressed in pre-eclampsia, but high plasma levels are also found in cancer and ischemia (86-88). Pre-eclampsia occurs during pregnancy and is characterized by hypertension and proteinurea, and it was suggested that these symptoms are caused by an abnormal suppression of VEGF-A signalling (88, 89). VEGFR1 can suppress VEGF-A-dependent signalling due to a higher binding affinity to VEGF-A than VEGFR2. Although VEGFR1 binds VEGF-A with a higher affinity, it induces only moderate signalling due to its weak kinase activity compared to that of VEGFR2 in endothelial cells (90). Thus, VEGFR1 can act as a natural inhibitor for VEGF-A, able to block VEGF-dependent signalling of VEGFR2 (91). The differences in kinase

activity between VEGFR1 and VEGFR2 following VEGF-A binding are due to sequence differences in both proteins (92-94). Both receptors can form heterodimers in response to VEGF-A in vitro, suggesting that this might be a mechanism for VEGFR1 to influence VEGFR2 signalling (95-97). Additional ligands of VEGFR1 are VEGF-B and PIGF. Regulation of inflammatory cell recruitment is mediated by PIGF, whereas VEGF-B has minor angiogenic roles in vivo but regulates the fatty-acid uptake in endothelial cells (98-100).

VEGFR3 (Flt-4) is expressed in blood vessels in early embryogenesis and it was demonstrated that mice lacking VEGFR3 expression die at E10.5 due to cardiovascular failure and disorganization of vasculature (101). In contrast, its expression is unique to lymphatic vessels in adults, suggesting its implication in the regulation of lymphogenesis (102). In endothelial cells, its expression is induced during active angiogenesis, for instance, in tumour blood vessels or in tip cells (16, 103). VEGFR3 only binds specifically to VEGF-C and -D and can form heterodimers with VEGFR2 necessary to induce VEGFR3 kinase activity and downstream signalling (figure 3) (104-107).

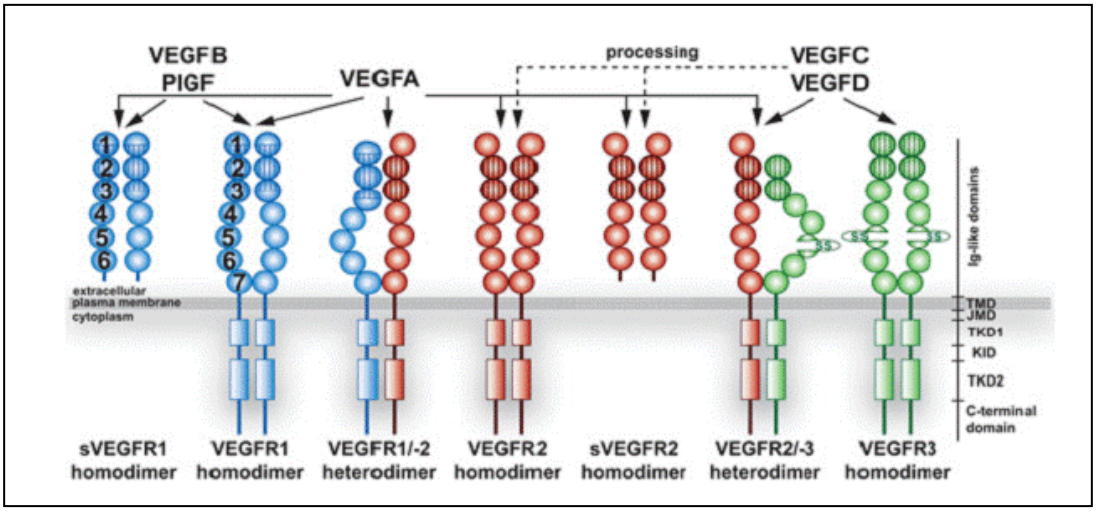

Figure 3: Different receptor forms of VEGFR and their specific ligands.

Schematic presentation of five VEGF isoforms with their specific binding to different VEGFR receptors, inducing homo- and heterodimerization. Soluble VEGFR1 and VEGFR2 lack the seventh Ig-like domain. Taken from (108).

1.4.1 VEGFR2 is the main mediator of the pro-angiogenic VEGF signalling

Although all three VEGF receptors are expressed during development, in vivo and in vitro studies demonstrated that VEGFR2 is the main receptor in adults, transmitting VEGF-dependent signalling. Upon VEGF binding to VEGR2, conformational changes in the receptor structure induce receptor dimerization (109). This dimerization is accompanied by the activation of its kinase activity and autophosphorylation on tyrosine residues in the C-terminal tail, creating docking sites for signalling molecules. Major phosphorylation sites of VEGFR2 are Y801 at the juxtamembrane domain, Y951/Y996, in the kinase insert domain, Y1054/1059, located in the autoactivation loop of VEGFR2, Y1175, and Y1214 in the C-terminal tail (figure 4). Additional phosphorylation sites are Y1223, Y1305, Y1309 and Y1319, but their function is still unclear (110, 111). Tyrosines 1054 and 1059 are located in the kinase activation loop of VEGFR2, and their phosphorylation is required for the induction of its kinase activity (112). Signal transducing molecules such as PLCγ, PI3K, AKT, TSAd, Shc, Grb2, Nck, SHP-2 and Src bind to the phosphorylated tyrosines of VEGFR2 via SH2-domains, inducing signalling cascades to promote biological functions of endothelial cells (113-117). For example, Src was reported to bind to phosphorylated Y1059 of VEGFR2, inducing phosphorylation of the VEGFR2 residue Y1175 (figure 4). This residue in turn serves as a binding site for PLCγ and induces cell proliferation (111). Src kinase activity is required for various ECs function including cell proliferation, survival, migration and vascular

permeability (114, 118). Thus, these data suggest a central role of Src in the mediation of angiogenic responses downstream of VEGFR2.

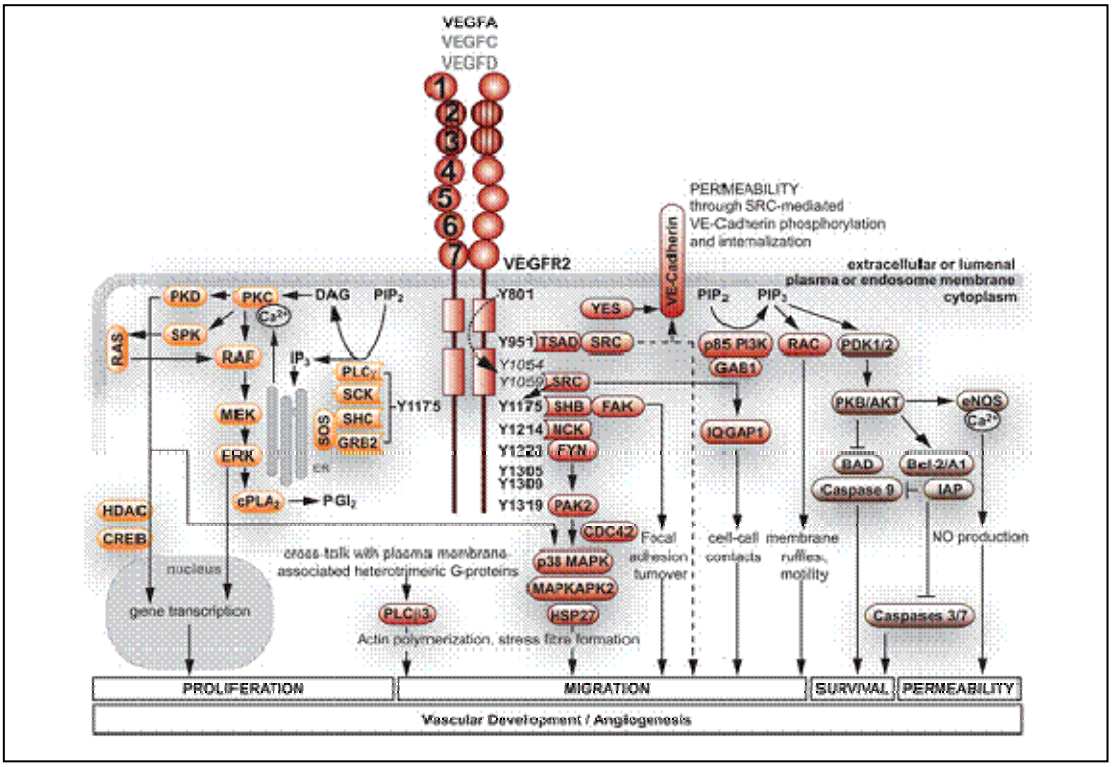

Figure 4: VEGFR2 and its downstream signalling.

The receptor VEGFR2 is localized at the cell membrane of EC. The dimerized VEGFR2 is active and its tyrosine phosphorylation sites are shown. Tyrosines 1054/1059 are important residues in the kinase autoactivation loop. Other main phosphorylation sites are Y801, Y951, Y1175 and Y1214. Signalling molecules bind to specific tyrosine residues and induce signalling cascades resulting in the establishment of various biological function of EC upon VEGF stimulation. Taken from (108).

1.4.1.1 Migration / Invasion

New vessel formation requires cell migration of endothelial cells (figure 5). Interestingly, several signalling pathways can induce VEGF-dependent cell

migration. Numerous studies demonstrated that VEGF-induced cell migration involves Src activity. For instance, SHB (SH2-domain-containing adaptor protein B) binds to Y1175 of VEGFR2 resulting in the activation of FAK, a central player in cell adhesion and migration (119, 120). In association with Src, the FAK-Src complex transmits signals from sites of ECM-cell contact into the cell, thereby influencing cytoskeleton remodelling and cell motility (figure 4) (121, 122). The binding of TSAd (T cell-specific adaptor molecule) to Y951 of VEGFR2 creates a binding site for Src, leading to Src activation (110, 123). Activated Src can induce the activation of Gab1, which in turn induces the PI3K pathway and the GTPase Rac to promote cell migration (110, 124, 125). In addition, Y1214 of VEGFR2 is also implicated in the mediation of cell migration via the binding of Nck and subsequent induction of Cdc42, p38 MAPK and PAK2 (126-128).

Invasion is an essential Src-dependent function of EC during new vessel formation downstream of VEGFR2 (figure 5) (129). Endothelial sprouting implicates the breakdown of the basement membrane by EC as one of the earliest steps. The induction of Matrix-metalloproteinases (MMPs) is essential for ECM degradation. For instance, it was shown that the JNK- and PI3K-pathways account for MT1-MMP induction in response to VEGF in rat EC, leading to ECM degradation (130). Moreover, MT1-MMP is involved in the invasion of EC during early events of angiogenic sprouting (131). Invasion can also be induced by angiogenic factors as well as biodynamic signals including blood flow (132, 133)

1.4.1.2 Vascular permeability

One of the key functions of VEGF is the induction of vascular permeability in ECs (figure 5). Several studies demonstrated that VEGFR2 mediates permeability in vivo (134). During angiogenic sprouting the remodelling of endothelial cell-cell junctions is one of the first steps, allowing subsequent induction of permeability. The entirety of in vitro and in vivo studies suggests a major role for Src in the mediation of

permeability (135, 136). Accordingly, vascular permeability is impaired in mice lacking Src expression (114). In response to VEGF, VE-cadherin, the major protein forming the adherens junctions, and β-catenin are phosphorylated in a Src-dependent manner (137-139). As a consequence of VE-cadherin and β-catenin phosphorylation, cell-cell contacts are loosened, impairing their integrity. Subsequently, the angiogenic sprout starts to invade the ECM to form a new capillary. Vascular permeability can also be induced by VEGF-mediated eNOS activation and NO production (140, 141). eNOS activity is stimulated by PLCγ-dependent Ca^{2+}-influx following Y801 phosphorylation or by its direct AKT-dependent phosphorylation on serine 1179 leading to increased permeability of endothelial cells (142, 143).

1.4.1.3 Proliferation

PLCγ is the major promoter of endothelial cell proliferation (figure 4). Upon VEGF stimulation, tyrosine residue 1175 of VEGFR2 is phosphorylated in a Src-dependent manner and recruits PLCγ to the receptor (144). The binding of PLCγ results in the induction of the Raf-MEK-MAPK-pathway via PKC and consequently in the proliferation of endothelial cells (111, 145). Additionally, endothelial cell proliferation is driven by the Src-mediated induction of the Ras-pathway and implicates the binding of Shc or Grb2 to Y1175 of VEGFR2 (146-148). Moreover, adherens junctions play a major role in the regulation of endothelial cell proliferation. The engagement of VE-cadherin mediates contact inhibition of endothelial cells via the dephosphorylation of VEGFR2 and ERK1/2. This process involves protein tyrosine phosphatases that colocalize with VE-cadherin and mediate the inhibition of cell proliferation (149, 150). Moreover, increasing amounts of Csk were shown to associate with VE-cadherins in cells at high density. Csk is a negative regulator of Src activity and mediates inhibition of cell proliferation under these conditions (151).

These data demonstrate that proliferation is tightly regulated in EC, implicating numerous mechanisms.

1.4.1.4 Survival

Endothelial cell survival is crucial to maintain blood vessel integrity and subsequently nutrition and oxygen supply for the organism (figure 5). PI3K/AKT is the main pathway promoting cell survival in response to VEGF (152). The adaptor protein Gab1 was shown to be involved in the promotion of cell survival in vitro and in vivo in response to VEGF (124, 153). Gab1 mediates the activation of PI3K, which in turn induces the generation of PIP3, leading to AKT activation and phosphorylation of its downstream target FOXO1/4 (154, 155). Moreover, AKT can directly phosphorylate BAD (Bcl-2 associated death promoter) and caspase 9 to inhibit apoptosis and promote cell survival (156, 157). The Src-FAK complex presents another pathway inducing cell survival. Upon VEGF stimulation, Src-dependent phosphorylation of Y861 of FAK is required for the inhibition of apoptosis, which is distinct from the P3K-AKT pathway (158). Interestingly, the interaction of VEGFR2 and VE-cadherin is essential to mediate PI3K-AKT-induced cell survival (159). Previous work in our laboratory showed that at adherens junctions activated Src induces the phosphorylation of Gab1 and the recruitment of PI3K to the complex, leading to the activation of AKT and FOXO1/4 and to the induction of cell survival in response to VEGF (160).

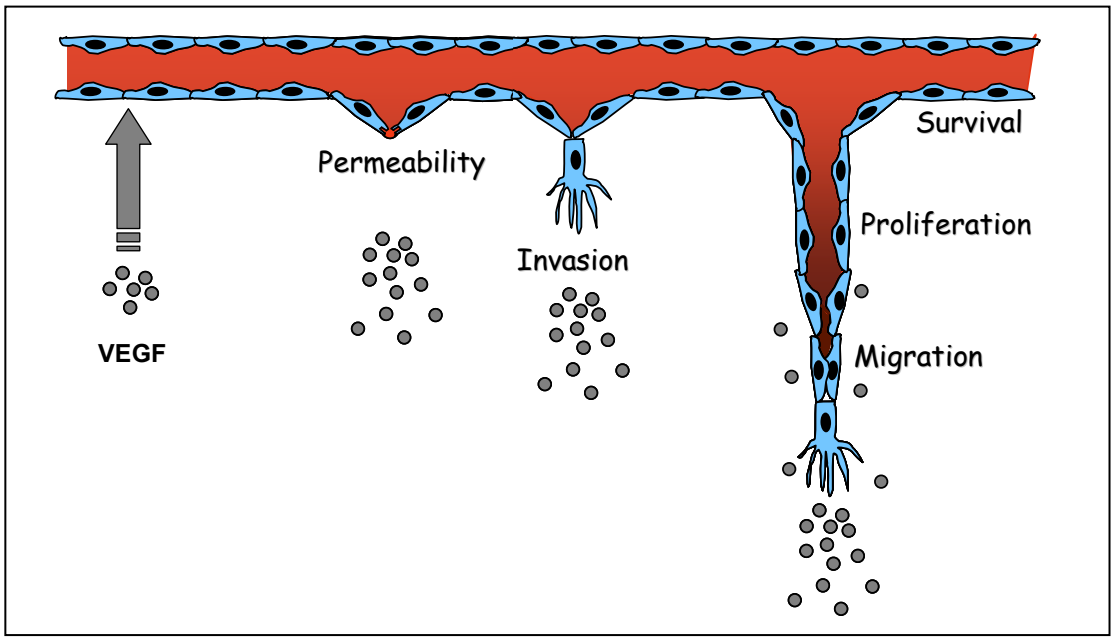

Figure 5: Endothelial cell functions that are implicated in capillary formation in response to VEGF.

Upon VEGF stimulation, endothelial cells loosen their cell-cell contacts to induce permeability and the degradation of the basal membrane. Specialized cells that can sense the pro-angiogenic gradient have high filopodia formation at the leading edge of the cell. These cells are characterized through high cell motility. Cells behind the high motile tip cells proliferate to elongate the newly formed sprout. Finally, the new sprout is stabilized by the recruitment of pericytes and VSMCs allowing the new vessel to mature. Quiescent EC are dense and form stable cell-cell contacts along with high cell survival.

1.5 Regulation of angiogenesis by protein tyrosine phosphatases (PTPs)

The dynamic equilibrium between activators and inhibitors of cellular signalling is essential for appropriate cellular functions. Receptor tyrosine kinases have crucial roles in the phosphorylation of tyrosine or serine residues and in the mediation of biological cell functions. The most important antagonists of receptor tyrosine kinases

are protein tyrosine phosphatases (PTPs), which negatively regulate induced cell signalling by the dephosphorylation of signal transducer molecules and growth factor receptors. They are involved in the regulation of numerous cell functions including proliferation, motility, cell-cell interactions, metabolism and immune response (161-165). Interestingly, many of the 38 classical PTP genes are present in EC, although their mRNA levels are low (166). The first protein tyrosine phosphatase was purified in 1988 by Tonks and colleagues approximately ten years after the discovery of tyrosine kinases (167). To date, the phosphatase family has enlarged to a structurally diverse and tightly regulated family. The human phosphatome encodes 107 PTP, which can be subdivided into several subfamilies based on structural and sequential diversity. The classic PTP family contains the receptor-like PTP (RPTP) and non-receptor cytoplasmic PTPs. Classic PTPs are specific towards tyrosine residues. The structure of RPTP includes a variable extracellular domain, a transmembrane domain and an intracellular domain containing in general two PTP domains (D1 and D2) with location of the catalytically active site in the D1 domain in most of the members (figure 6). The RPTP family can be subdivided into eight families (R1-R8) based on sequence similarities among the catalytic domains of these PTPs (168, 169). The members of the R1, R2 and the R4-R8 subfamilies contain two catalytic domains. In contrast to the R3 family of PTPs including DEP-1, VE-PTP, GLEPP1 and SAP-1 contains only one catalytic domain. The extracellular domain of RPTPs is often composed of Ig-like or fibronectin type III domains similar to cell adhesion molecules, suggesting the implication in processes that involve cell-cell or cell-matrix contacts (170).

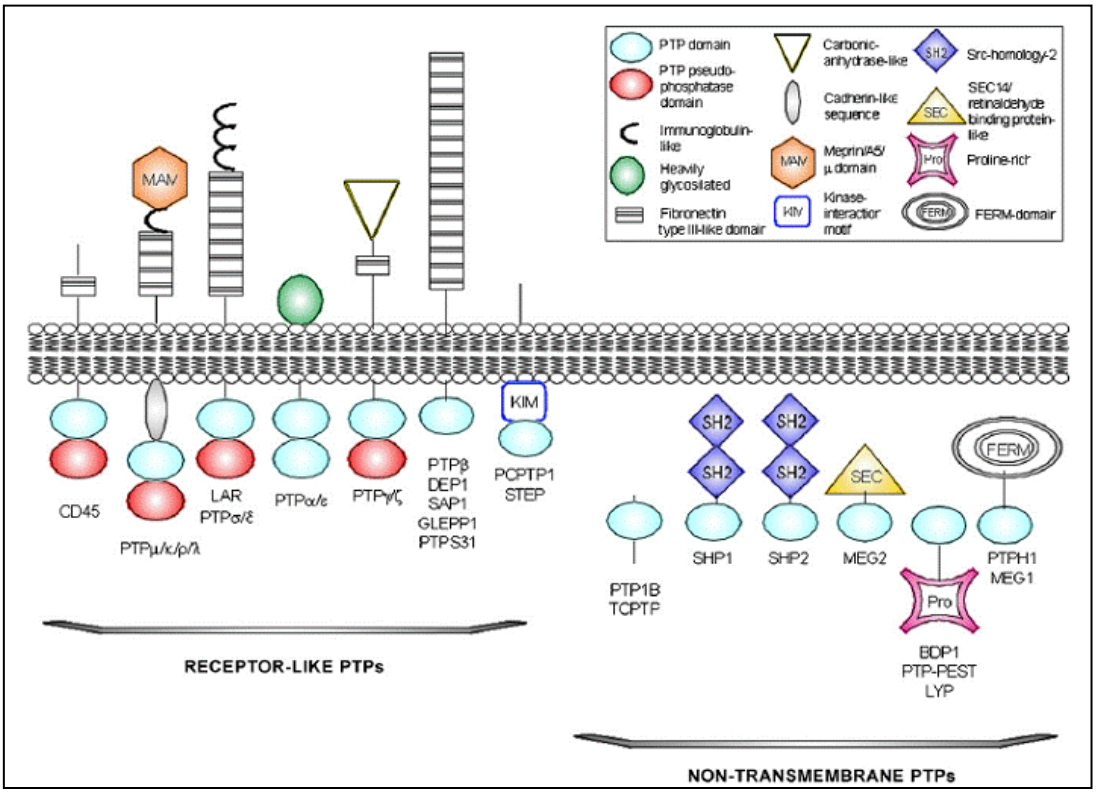

Figure 6: The family of classic PTPs.

Classic PTPs can be divided into receptor-like PTPs (RPTPs) and non-receptor PTPs. The R3 family of the RPTPs includes PTPs such as GLEPP1 (PTPRO), SAP1 and DEP-1 (PTPRJ), which are characterized by a large extracellular domain composed of eight fibronectin III motifs and a single catalytic domain with a small C-terminal tail. Adapted from (171).

The phosphatase activity domain contains a highly conserved cysteine residue responsible for the reaction of dephosphorylation by acting as a nucleophile attractant for the substrates. The two-step mechanism of dephosphorylation is highly specific towards the respective substrates and involves the formation of a cysteinyl-phosphate intermediate (figure 7). The reaction finally terminates with the hydrolysis of the intermediate resulting in the dephosphorylated protein (172). Even if the substrate specificity is mainly defined by phosphotyrosine of the substrate and specific

phosphotyrosine flanking sequences, recent studies also demonstrate a contribution of substrate-PTP binding domains in the target selection (173-175).

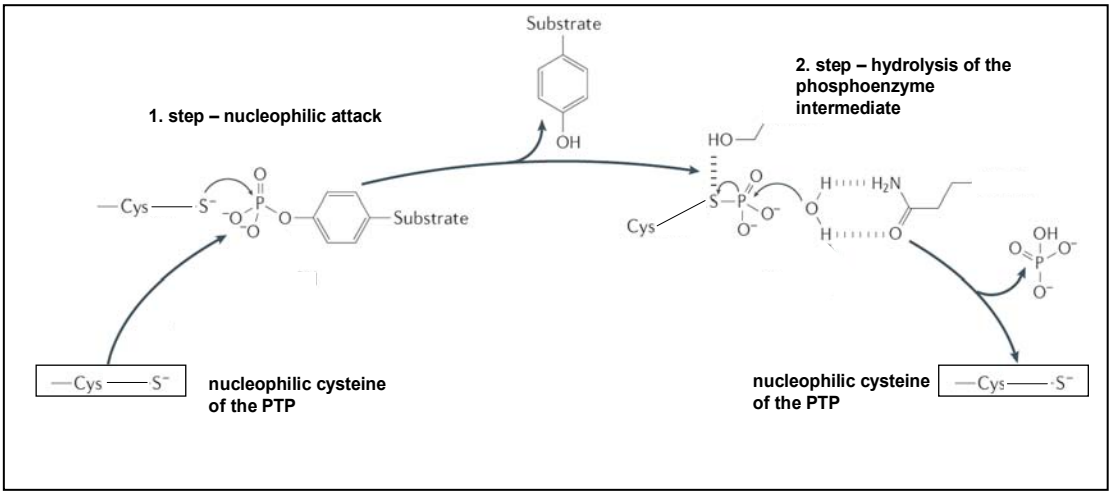

Figure 7: The catalytic mechanism of PTPs.

The nucleophilic cysteine residue recognizes the phosphate of the target protein. The first step of the catalysis involves the nucleophilic attack on the phosphorylated target protein to form a phosphoenzyme intermediate. The second step involves the hydrolysis of the intermediate. Finally, the substrate is dephosphorylated and the nucleophilic cysteine restored for a new reaction. Adapted from (170).

1.5.1 Regulatory mechanisms of classic PTP activity

PTPs are implicated in the mediation of different cell functions including growth, motility and immune response (162-165). In order that PTPs can tightly regulate RTK, they have to be precisely regulated by several mechanisms. For instance, substrate specificity of PTPs is regulated by the catalytic domain and substrate-specific sequences. Moreover, other mechanisms including subcellular localisation and expression level of PTPs, post-translational modifications such as phosphorylation and oxidation are involved in the regulation of PTP activity.

Especially for RPTPs, dimerization and ligand binding are important processes to regulate their activity.

1.5.1.1 Dimerization as a mean to control PTP activity

Receptor-like PTPs such as PTPα, PTPε, PTPδ, LAR, CD45 can form dimers (176-178). PTP can form homo-or heterodimers. Several PTPs such as PTPα, CD45 and DEP-1 can generate homodimers in the membrane by the interactions of transmembrane domains as has already been shown for receptor dimerization (179-184). Interestingly, the PTPμ forms homodimers via its extracellular domain and the dimer length is similar to the width of adherens junctions connecting two cells, suggesting that PTPμ homodimers control the distance between cells (185-187). However, homodimers can also be formed by the interaction of catalytic domains of PTPs. The interaction mechanism of the catalytic domain is explained with the "wedge-wedge" model. This model suggests that the N-terminal helix-turn-helix wedge motif binds to the catalytic domain of the other interacting monomer, leading to dimerization of the complex and PTP inactivation. This is the case for the homodimer formation of PTPα and for the heterodimer CD45-EGFR upon EGF stimulation (188-190). Hence, dimerization can inactivate PTPs, but there is also evidence that active dimers exist. For instance, DEP-1 was reported to form active dimers following binding with a bivalent anti-DEP-1 antibody. In contrast, the same study showed that the binding with a monovalent antibody failed to induce DEP-1 dimerization. Using the bivalent antibody results in the increase of DEP-1 activity and subsequently the inhibition of cell growth in vitro and the blocking of angiogenesis in vivo (191). Recently, it was proposed that the DEP-1 catalytic domain could form dimers in solution similar to the inhibitory wedge-wedge model proposed for PTPα, resulting in the inhibition of DEP-1 activity (192). But, notably, it was not shown if DEP-1 dimerizes in this manner in living cells. In contrast to PTPα,

DEP-1 and other members of the RPTP R3 family such as SAP-1 contain only one catalytic domain and SAP-1 was shown to dimerize via its extracellular rather than the catalytic domain (174). Another study demonstrated that DEP-1 dimerization can be induced by H_2O_2 or by the cross-linking agent BS^3, leading to the formation of disulphide bonds in the extracellular domain (184).

1.5.1.2 Ligands as regulators of PTP activity

Due to the large diversity of the extracellular domain structure, the number of potential ligands is huge. Most RPTPs have more than one ligand. Certain PTPs can be a ligand for themselves, resulting in the dimerization of two PTP monomers. This was shown for PTPμ, PTPλ as well as PTPκ (193-195). Moreover, during development in Drosophila PTP-LAR-mediated functions are regulated by two high affinity ligands (196). A previous study demonstrated that a component in the extracellular matrix leads to the activation of DEP-1, indicating the presence of a potential ligand for DEP-1 in the ECM (197). Recently, syndecan-2 was identified as a ligand that activates DEP-1 and promotes the dephosphorylation of PI3K in fibroblasts. This process implicates Src activity to finally transmit the signal for the promotion of β1 integrin-dependent adhesion (198). Moreover, another recent study described soluble Thrombospondin-1 (TSP-1) as a high specific ligand for DEP-1, increasing its catalytic activity. TSP-1 binds to the extracellular domain of DEP-1, suggesting that DEP-1 acts as receptor for TSP-1 mediating EC growth inhibition (199).

1.5.1.3 Post-translational modification: Phosphorylation on tyrosine and serine residues

Phosphorylation is considered as a regulatory mechanism of protein activity and was proposed to also be a regulatory mechanism for PTP activity. Phosphorylation regulates the intramolecular conformation of PTPs as well as their interaction with other proteins.

Tyrosine phosphorylation is commonly reported to activate PTPs and/or to direct them selectively to specific substrates. Several PTPs such as CD45, PTPα, SHP-1 and SHP-2 were reported to be phosphorylated on tyrosine, leading to higher PTP activity in different conditions (200-202). For instance, observations in NIH3T3 cells demonstrated that 20% of PTPα is phosphorylated on Y789 and bound to Grb2. Previous reports demonstrated that in basal conditions Grb2 binding to Y789 competes with Src binding to this residue, circumventing Src activation. During mitosis, the affinity of Y789 for the SH2 domain of Grb2 was reported to decrease, leading to its dissociation from PTPα. Y789 is now accessible for Src binding and Src is subsequently activated by PTPα. In contrast, Vacaru and colleagues observed recently that Grb2 does not dissociate from Y789 during mitosis. Consequently, the authors showed that Src binding to PTPα is not dependent on Y789 (203-206). The same study demonstrated that serine 204 of PTPα is dephosphorylated during mitosis, leading to increased Src binding to PTPα and subsequent Src activation (206). Interestingly, CD45 can regulate PTPα activity in T cells through the dephosphorylation of PTPα Y789 increasing Fyn activity and Pyk2 phosphorylation in T cells (207). This study provided evidence that PTPs can regulate the catalytic activity of another PTP, describing an additional regulatory level of PTP activity regulation. CD45 was reported to be also phosphorylated on serine residues. However, the impact of serine phosphorylation on CD45 activity is poorly defined. For instance, Phorbolester treatment induced the phosphorylation of two serine residues in lymphocytes, nonetheless no notable changes in the catalytic activity of CD45 were observed under these conditions (208). In the other hand, it was reported that the loss of serine phosphorylation decreases CD45 catalytic activity (209).

Certain PTPs of the R3 family including VE-PTP, GLEPP1 and SAP-1 are phosphorylated in a Fyn-dependent manner in their C-terminal tail, allowing the association with Grb2 and Fyn. While the functional significance for the binding with Grb2 remains unclear, the association of Fyn was proposed to be important for Fyn activation. Moreover, co-expression of SAP-1, GLEPP1 and VE-PTP with activated Ras leads to induction of cell spreading and lamellipodia formation in CHO cells (210). DEP-1, which is also part of the R3 family, was shown to be phosphorylated on tyrosine in several breast cancer cell lines following pervanadate treatment (211). Further analysis in the same study showed that a serine/threonine kinase is associated with DEP-1 and could phosphorylate DEP-1 in the C-terminal tail. Another study found that active Src stimulates DEP-1 activity via direct association and phosphorylation in a somatostatin-dependent manner (212). In HeLa cells, weak EGF-induced tyrosine phosphorylation of the catalytically inactive DEP-1 D/A mutant was observed, confirming previous reports that DEP-1 can be phosphorylated on tyrosine (213). Nevertheless, these studies did not answer the question whether endogenous DEP-1 is phosphorylated in physiological conditions, which residues are phosphorylated and if DEP-1 phosphorylation has an impact on DEP-1 activity, cell signalling and cell function.

1.5.1.4 Post-translational modification: Oxidation

Reversible oxidation of the cysteine in the catalytic site negatively regulates PTP activity. Due to a low pKα, the conserved cysteine usually exists as a thiolate anion at physiological pH in cells and is highly susceptible to oxidation (214, 215). Production of reactive oxygen species (ROS) is driven by NADPH oxidase, but also indirectly in response to various cellular stimuli by a number of metabolic processes as by cyclooxygenases (COX), lipoxygenases (LipOX) and mitochondrial respiration, nitric oxide synthases and xanthine synthases. Recently, it was shown that peroxidized lipids can act as inducer of PTP oxidation (216). ROS transiently convert the active

cysteine site into either sulphenic, sulphinic or sulphonic acid (figure 8) (217-219). For the reaction to be reversible, the cysteine should not oxidize further than to sulphenic acid, otherwise the oxidation is irreversible and the PTP remains inactivated (figure 8). For instance, PTP1B oxidation to sulphenic acid is accompanied by a conformational change of the active site and disrupts the interaction with its substrate to finally expose the oxidized cysteine to the cell environment (220). This conformational change avoids irreversible oxidation and complete inhibition of PTP activity, so that the PTP can be reduced to restore the active form. In response to PDGF, the formation of an intramolecular disulphide bond protects the catalytic cysteine of LW PTP from irreversible oxidation (221). A recent study demonstrated that DEP-1 could be oxidized in cultured cells following induction of hypoxia. In the same study, results suggested that increased PTP oxidation correlated with decreased PTP activity when cells were exposed to hypoxic conditions followed by reoxygenation (222). Godfrey and colleagues demonstrated that DEP-1 is inactivated by oxidation in FLT3-ITD-induced AML, leading to the induction of FLT3-ITD-dependent cell transformation (223).

Thus, oxidation represents an important mechanism to regulate PTP activity in a time-dependent manner via a rapid reversible inhibition of PTP activity to allow transmission of cell signalling events.

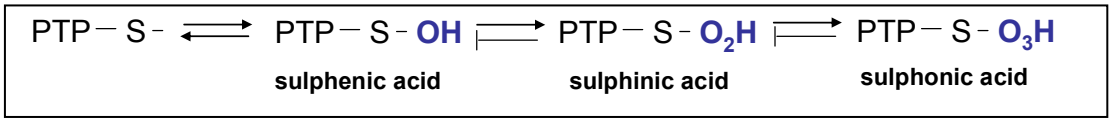

Figure 8: Reversible inactivation of PTPs through oxidation.

The oxidized catalytic cysteine has impaired nucleophilic function leading to the reversible inactivation of the PTP. The rapid conversion of the sulphenic form of the oxidized cysteine to a cyclic sulphenamide protects the catalytic cysteine from being further oxidized into the irreversible forms sulphinic or sulphonic acid.

1.5.1.5 Subcellular localisation

Many PTPs display high activity, which is tightly controlled by different mechanisms. The subcellular localisation of PTPs defines the access to PTP substrates and is a manner to control PTP activity. Different domains including PDZ, FERM, SH2, proline-rich domains and Sec14 allow PTPs to localise to the appropriate cell compartment. For instance, SH2 domains allow that PTPs such as SHP-1 and SHP-2 are localised at the cell membrane, where they interact with RTK or regulate tyrosine phosphorylation in the context of cell-cell or cell-matrix adhesion (224, 225). PDZ and FERM domains as well as proline-rich domains allow PTPs to bind to their substrates. For instance, PTP-BL is recruited via a FERM domain to the apical site of epithelial cells, regulating its catalytic and substrate specificity (226). In addition, the Sec14-homology domain targets PTP-MEG2 specifically to secretory vesicles to regulate vesicle fusion (227). Furthermore, specific target sequences can direct PTPs to their compartment. For instance, a hydrophobic sequence, which anchors PTP1B and TC-PTP to the ER, contributes to limited substrate recognition (228-230). Lastly, nuclear localisation signals in PTPs exist to direct them to the nucleus as it was shown for the alternatively spliced form of TC-PTP (231). Interestingly, deletion of the DEP-1 extracellular domain prevents its localisation at the membrane and induces the formation of disulphide bond-dependent higher order complexes in the intracellular compartment that are unable to block cell proliferation. These results suggest that the localisation of DEP-1 at the plasma membrane is important to mediate cellular functions as the growth arrest (184). Hence, the presented examples demonstrate an essential contribution of the localisation factor to regulate the availability of substrates and to define PTP specificity.

1.5.1.6 Expression

An obvious regulatory mechanism of proteins is their expression level. Thus, it is not surprising that PTPs can be regulated by their expression level. Several PTPs are ubiquitously expressed such as PTP1B, DEP-1 and SHP-2. However, there are also examples of PTPs whose expression is limited to specific tissues or cell types such as PTPH1 expression to neuronal tissue and VE-PTP to endothelial cells (232, 233). Several mechanisms were described regulating the mRNA expression of specific PTPs. A number of PTPs including DEP-1, VE-PTP and PTPμ are upregulated when cells reach confluence, proposing a role in the regulation of cell growth (234-236). Several studies demonstrated that different expression levels of CD45 have different effects on T cell signalling. High expression above physiological levels was demonstrated to inhibit T cell signalling, while small amounts of CD45 are sufficient to rescue inducible TCR signalling in $CD45^{-/-}$ mice (237). Interestingly, the activation of the SFK family member Lck is impaired in T cells expressing high levels of CD45 due to the dephosphorylation of the inhibitory Y505 and activatory Y394 of Lck. In contrast, lower levels of CD45 only promote the dephosphorylation of the inhibitory Y505 Lck, leading to Lck activation (238). Thus, different expression levels of CD45 are required to tightly balance TCR signalling and T cell development via the regulation of Lck activity. Additionally, alternative use of PTP gene promoters leads to tissue-specific PTP mRNA expression, as described for SHP-1, or the expression of different PTP isoforms as described for PTPε (239, 240). The regulation of PTP mRNA allows the control of PTP expression at different levels concomitant with the access limitation to specific PTP substrates.

1.5.1.7 Proteolytic cleavage

Certain PTPs can be regulated by limited proteolysis to control PTP activity and function. This mechanism is described for several members of RPTP including PTPμ, PTPλ, PTPδ and PTPκ. The extracellular domain is the subject of proteolytic cleavage by proteinases/convertases during PTP biosynthesis. This modification leads to a non-covalent association of the extracellular to the transmembrane and intracellular domains in mature PTPs (241, 242). Additional cleavage enzymes such as ADAM10, γ-secretase and calpain contribute to further cleavage of mature PTPs, defining the cellular redistribution that can be associated with changes in PTP activity (243, 244). For instance, calpain-mediated cleavage of PTP1B leads to its relocalisation from the plasma membrane to the cytosol, which is accompanied by an increase in PTP1B activity (245). Calpain also promotes PTPα cleavage at the intracellular domain, resulting in PTPα translocation away from the membrane and limiting the access to its substrate Src (246).

1.6 Regulation of VEGFR2-dependent signalling and biological functions by PTPs

Based on the crucial role of VEGFR2 in the mediation of angiogenic responses, it is important to define molecular mechanisms implicated in the regulation of VEGFR2. Maintenance and integrity of the endothelial cell barrier is crucial to sustain functional blood vessels and cell-cell contacts. Adherens junctions were shown to be especially important for this. The major protein in adherens junctions is VE-cadherin (247). Additionally, various proteins are associated with VE-cadherin in adherens junctions including β-catenin, plakoglobin, p120 catenin, Src and Csk (figure 9) (151,

248). The VE-cadherin complex was proposed to relate VEGFR2 to junction-associated PTPs. To date a number of PTPs were identified that are able to dephosphorylate VEGFR2 including SHP-2, PTP1B, VE-PTP, PTPμ, TC-PTP and DEP-1. PTP-mediated regulation of VEGFR2 was proposed to be important for maintaining low tyrosine phosphorylation levels at adherens junctions to ensure cell-cell contact homeostasis and integrity of the endothelial cell barrier. For instance, the site-specific dephosphorylation of VEGFR2 by PTPs mediates the inhibition of cell proliferation (149, 249-253). Silencing of PTPμ, TC-PTP and PTP1B leads to enhanced vascular permeability, demonstrating their negative role on VEGFR2 signalling and the phosphorylation of junctional proteins (250, 254, 255). Moreover, the dissociation of VE-PTP and SHP-2 from the VE-cadherin complex is necessary to increase vascular permeability, suggesting that they inhibit VEGFR2 signalling (224, 256). Different extracellular matrices can influence the function of PTPs at sites of cell contacts. For instance, TC-PTP and VE-PTP mediate different biological outcomes in endothelial cells dependent on which extracellular matrix was used to culture these cells. VE-PTP expression is upregulated in endothelial cells cultured on a three dimensional collagen gel and mediates the formation of tubular structures, while endothelial cells cultured on fibronectin do not increase VE-PTP expression but induce proliferation (252). Although several PTPs are implicated in the dephosphorylation of VEGFR2, the impact on VEGFR2 signalling at cell contacts is dependent on PTP localisation to these sites, the response to different type of extracellular matrix and the angiogenic stimulus.

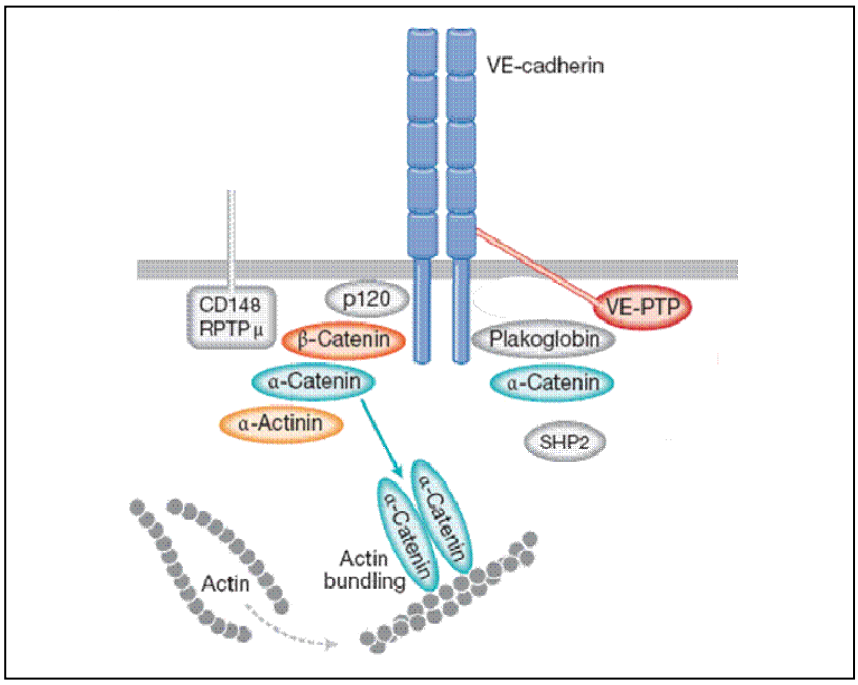

Figure 9: The adherens junction complex with the associated proteins and the associated PTPs DEP-1 (CD148), PTPμ and VE-PTP.

In confluent endothelial cells, VE-cadherin is clustered to form adherens junctions. VE-cadherin molecules can form these intercellular connections through the homophilic binding to another VE-cadherin monomer of a neighbouring cell. The cytoplasmic tail of VE-cadherin molecules is bound to the cytoskeleton via various connecting protein such as p120 catenin, plakoglobin and α-,β-,γ-catenin. Commonly, PTPs as VE-PTP and DEP-1 but also RTK as VEGFR2 are localised in this complex. Adapted from (257).

1.6.1 VE-PTP/PTPRB

VE-PTP$^{-/-}$ mice die at embryonic day 10 due to abnormal angiogenesis the failure to remodel the primitive vascular network especially in the yolk sac (233, 258). Interestingly, VE-PTP expression is limited to endothelial cells and increases with cell density, suggesting a function in cell contact inhibition (233, 235, 259). Accordingly, VE-PTP can dephosphorylate VEGFR2, leading to the inhibition of VEGFR2 signalling in resting cells. Under these conditions, VE-PTP is also

associated with VE-cadherin, mediating cell contact adhesion and the maintenance of EC contact integrity in cooperation with plakoglobin (252, 260). In contrast, in VEGF-stimulated cells VE-PTP association with VEGFR2 and VE-cadherin is lost, thus permitting VEGF-dependent signalling as well as phosphorylation of VE-cadherin and associated junctional proteins. The VEGFR2-VE-PTP complex is reconstituted with time and consequently leads to VEGFR2 dephosphorylation and deactivation (252). These data demonstrated that VE-PTP exerts its role in EC contact maintenance, while its dissociation from cell contacts is essential to induce VEGFR2-dependent signalling and endothelial cell functions such as vascular permeability during angiogenesis (256, 260).

1.6.2 SHP-2/PTPN11

The implication of SHP-2 in the regulation of VEGFR2 signalling is well known. It was shown that the important negative Src regulator Csk associates with Y685 of VE-cadherin at cell contacts (151). Moreover, SHP-2 was shown to be recruited to VE-cadherins in response to VEGF and to dephosphorylate the Csk-binding site Y685 of VE-cadherin. The dissociation of Csk from VE-cadherins decreases the phosphorylation of Src Y529, promoting its activation (261). Thus, SHP-2 can control the phosphorylation of the inhibitory tyrosine Y529 of Src in an indirect manner through the regulation of Csk availability at endothelial cell contacts in response to VEGF.

1.6.3 DEP-1/CD148/PTPRJ

DEP-1 is a member of the RPTP family which was first described in 1994 by Östman et al. and which is expressed in many cell types including EC, haematopoietic and epithelial cells (234, 262-264). It is composed of a large extracellular domain containing eight fibronectin III motifs, a transmembrane domain and a single intracellular domain containing the unique phosphatase catalytic motif and a small C-terminal tail. Expression levels were reported to increase when cells reach high density, suggesting its implication in the mediation of contact inhibition (234). DEP-1 expression is also observed in early zebrafish and mouse embryos as well as in C.elegans, indicating that DEP-1 already has a role in evolutionary lower organisms such as nematodes (265-267). Moreover, in zebrafish embryos DEP-1 expression is especially observed in the neuronal tube, in muscles and endothelial cells (268).

Various lines of evidence suggest a role for DEP-1 in the regulation of angiogenesis. In 2003, a DEP-1 knock-in mouse model showed that the replacement of the intracellular domain of DEP-1 with GFP abrogates the remodelling of the primitive vascular network and interferes with the viability of embryos at embryonic day 10.5. During early development, homozygous mouse embryos display higher numbers of EC in the yolk sac, indicating increased proliferation. Vessels in the yolk sac are enlarged and less branched. In these mice the dorsal aorta is narrowed and the mice show severe abnormalities in the heart (265). Thus, DEP-1 function is indispensable for angiogenic processes during development and DEP-1 has a positive role on remodelling and ramification of newly formed vessels. Interestingly, consistent with defects in vasculature seen in the DEP-1 knock-in mouse model during development, VE-cadherin knockout mice show a similar phenotype with severe vascular defects and embryonic lethality (269, 270). In contrast, a study demonstrated that the deletion of exon 3, 4 and 5 of DEP-1 gene results in mice

which have a null mutation in this specific locus. The knockout of DEP-1 exerts no effect on the vasculature and mice are perfectly viable and fertile (271). Consistent with this in vivo study, another mouse model in which the DEP-1 transmembrane domain was deleted (DEP-$1^{TM-/TM-}$), supports the findings that aberrant deletion of DEP-1 does not affect the viability of mice (272). However, analysis of the mouse immune system of homozygous DEP-$1^{TM-/TM-}$ mice revealed that peripheral B cell development is partially blocked similarly to what was observed for the $CD45^{-/-}$ mouse model (273). Therefore, these data suggest that CD45 and DEP-1 have redundant roles in B cell development since the double mutation CD45 and DEP-1 severely affects B cell and myeloid development as well as immune receptor signalling. In immune cells of the double mutant, SFK are not activated due to elevated phosphorylation of Y529 (272). Further evidence that DEP-1 is implicated in the regulation of angiogenesis was shown during zebrafish development. In this model, DEP-1 is implicated in cell differentiation of arterial and venous EC whereas DEP-1 promotes the specification of arterial ECs. Experiments in DEP-1 knockdown zebrafish revealed that DEP-1 seems to be important for blood circulation. The study showed that blood flow is impaired due to the reduction of arterial differentiation, which is accompanied by an expanded expression of venous cell markers (268).

In cells, DEP-1 localizes in proximity to VEGFR2 and VE-cadherins at cell contacts where it binds to p120 catenin, β-catenin and plakoglobin (274, 275). The formation of the β-catenin/VE-cadherin/p120 catenin complex was proposed to link VEGFR2 to DEP-1 (figure 10). DEP-1 accomplishes its important role in VE-cadherin-mediated contact inhibition in endothelial cells through the dephosphorylation of VEGFR2 concomitant with inactivation of the ERK1/2 pathway (149, 159, 262). The decreased clustering of VE-cadherin along with dissociation of DEP-1 from VE-cadherin in sparse cells leads to higher VEGFR2 phosphorylation and higher VEGFR2 endocytosis in intracellular compartments. Endocytosed VEGFR2 forms particular signalling complexes to promote cell proliferation (150). In sparse cells, VEGFR2 promotes proliferation through MAPK

signalling, whereas in confluent cells it induces cell survival through the activation of the PI3K/AKT pathway (149, 159). The binding of VEGFR2 to VE-cadherin increases in confluent cells and concomitantly with DEP-1 expression as shown in a VEC positive cell model (149). In agreement with this, DEP-1 was shown to colocalise with VE-cadherins in double-labelled EC, indicating its accumulation at sites of cell-cell contact and suggesting that VE-cadherin expression defines the quantity of DEP-1 protein at these sites (191). These results suggest that DEP-1 expression and localisation in adherens junctions is marginal in sparse cells in contrast to confluent EC. Thus, DEP-1 expression levels are dependent on VE-cadherin expression levels and in turn partially dictated by cell confluence.

DEP-1 expression is decreased in vascular smooth muscle cells (VSMCs) after vessel injury. The same study showed that, downregulation of DEP-1 expression in VSMCs and MEF cells is concomitant with extensive PDGFR phosphorylation, proliferation and chemotaxis in the intima (276). In accord with this study, DEP-1 expression was reported to decrease in migrating and proliferating endothelial cells during vessel repair in vivo (262). Thus, these studies suggest that reduced DEP-1 expression allows growth factor-induced proliferation and migration and support the fact that DEP-1 function in endothelial cells is correlated with its expression level.

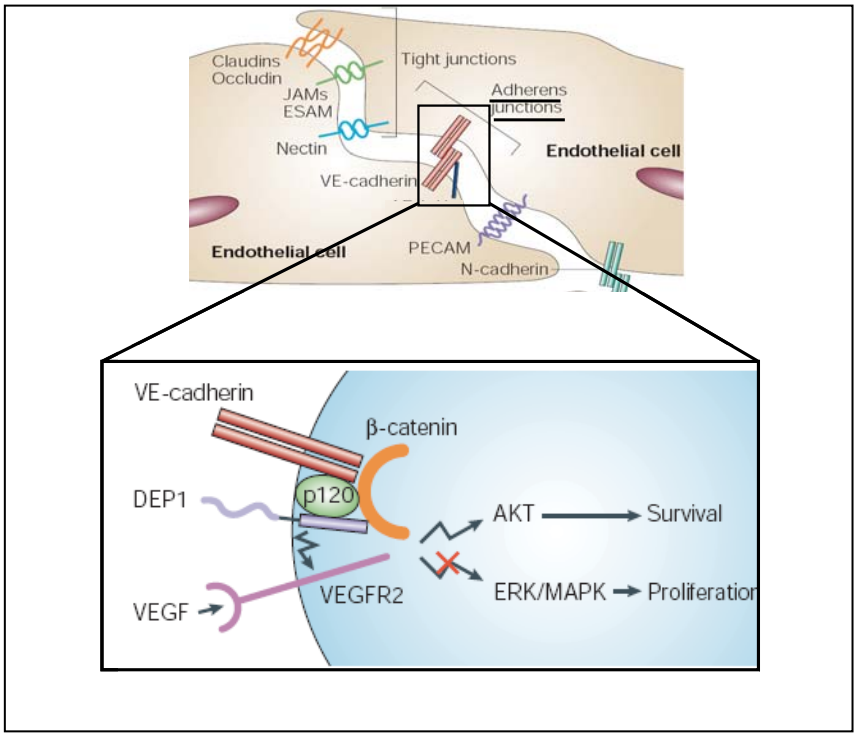

Figure 10 : Structure of adherens junctions and modulation of VEGFR2 signalling by VE-cadherin and associated proteins.

The association of VEGFR2 with VE-cadherins is known to influence VEGFR2 signalling upon VEGF stimulation at sites of cell-cell contact. In confluent cells, the VE-cadherins are clustered and associated with VEGFR2 at adherens junctions. Based on the model presented by Lampugnani and colleagues the receptor VEGR2 in collaboration with DEP-1 promotes cell survival under these conditions. In contrast to confluent cells, in sparse cells the clustering of VE-cadherins is reduced and the receptor VEGFR2 signals preferentially to promote cell proliferation. Adapted from (277).

1.7 Src family kinases (SFK) as major substrates of PTPs

Src and Src family kinases (SFK) are mediators of many cell functions including differentiation, cell motility, cell survival and proliferation and they have a central role in transmission of cellular signals to downstream effectors. Tight regulation of Src activity is accomplished by many PTPs.

1.7.1 Structure and regulation of Src activity

The tyrosine kinase Src contains numerous domains including an N-terminal carbon-myristoyl group linked to a SH4- and SH3-domain followed by a SH2-domain, the tyrosine kinase domain and the C-terminal tail (278). Two tyrosine residues are essential for the regulation of Src kinase activity (figure 11). In the inactive state, the phosphorylated inhibitory tyrosine 529 is linked intramolecularly to the SH2-domain, keeping Src in a closed conformation. When the inhibitory tyrosine 529 is dephosphorylated, Src adopts an open conformation, allowing the autophosphorylation of the activatory tyrosine 418 in the activation loop, thereby keeping Y418 accessible (279). Under basal conditions the major Src pool is phosphorylated on Y529 catalyzed by C-terminal Src kinase (Csk) (204). Both enzymes are expressed ubiquitously. However, Csk homology kinase (Csh) phosphorylates Src on Y529 as Csk does, with the difference that Chk expression is restricted to specialized tissues such as breast, testis, hematopoietic cells and neurons (278). Triggered by phosphorylation of another tyrosine in the SH2 domain (Y213), Src activation is slightly increased following PDGF stimulation. The interaction of Y529 with the SH2 domain is limited by phosphorylation of Y213, representing another Src regulation level. However, this residue has a marginal impact on the regulation of Src activity (280). Based on crystallization studies elucidating Src's 3-D-structure, the SH2 and SH3 domain of Src were shown to be implicated in the conformational change of Src upon stimulation, allowing dephosphorylation of Y529 and Y418 phosphorylation.

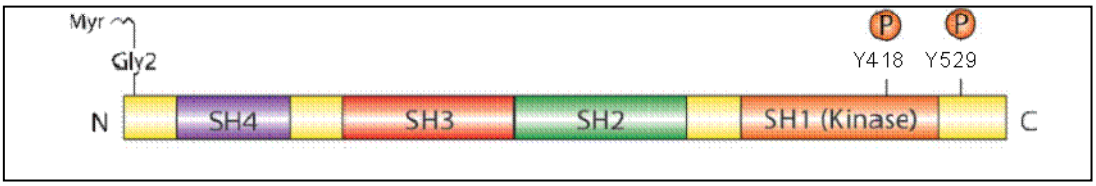

Figure 11 : Structure of Src and major tyrosine residues.

Src is composed of one SH2, SH3, SH4 and one kinase domain. The activatory tyrosine 418 (Y418) is localized in the activation loop and the inhibitory Y529 is found in the C-terminal tail of Src. Adapted from (281).

The SH2 and the SH3 domains contribute to intramolecular interactions mediating conformational changes accompanying various activation states of Src. To keep Src in an inactive state the Y529 in the C-terminal binds to its SH2 domain. Additionally, its SH3 domain is attached to a proline-rich motif in the linker domain to lock Src in the closed conformation (282, 283). Following activatory stimuli, Y529 dissociates from the SH2 domain. Tyrosine phosphorylated proteins or proline-rich-motif bearing proteins can compete with Src SH2 and SH3 domain binding, resulting in conformational changes and potential activation of Src (284). For instance, phosphorylated PDGFR recruits and activates Src via the interaction with Src SH2 domain (285, 286). However, to reach maximal Src activation, Y418 phosphorylation seems to be the most important event since Y418 phosphorylation was shown to block Csk mediated Src inactivation (287). Interestingly, recent studies illustrated a complementary regulatory mechanism for Src activation. ROS were shown to be implicated in the regulation of Src activity. Based on a thiol reaction, oxidation of cysteine residues results in the increase of Src kinase activity due to the induction of Y418 phosphorylation. In fibroblasts, ROS are required to fully activate Src in response to ECM-induced integrin engagement (288). Treatment with the ROS scavenger N-acetyl cysteine (NAC) decreases Src phosphorylation on Y418, leading to its localisation to endolysosomal compartments in human cancer cell lines (289).

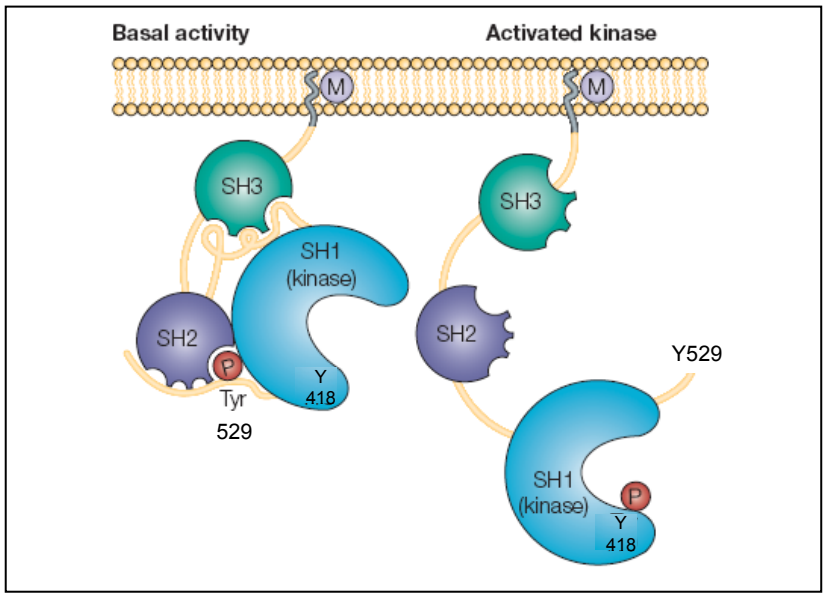

Figure 12: Conformational changes of Src are associated with its activation.

In basal conditions, Src is in a closed conformation. The phosphorylated inhibitory tyrosine Y529 is bound intramolecularly to the SH2 domain, while the SH3 domain is bound to the linker domain situated between the SH2 and the catalytic domain of Src. Following activatory stimuli, the Y529 is detached from the SH2 domain of Src allowing an open conformation. The activatory tyrosine Y418 is accessible for phosphorylation resulting in an optimal Src activation. Adapted from (290).

1.7.2 The role of PTP in the regulation of SFK

The best described mechanism promoting Src activation is the dephosphorylation of the inhibitory tyrosine 529 in the C-terminal tail (291). Its dephosphorylation contributes to the release of the Src SH2 domain and allows the opening of its conformation. Depending on the cell system and the stimuli, different PTPs may be involved in this process. PTPs can act on both Src tyrosine residues; however, most PTPs preferentially act on Y529.

1.7.2.1 PTPα

PTPα is a member of the RPTP containing a short extracellular and two intracellular catalytic domains (292). It is the first PTP shown to regulate Src activity and it was first described in fibroblasts from PTPα$^{-/-}$ mice. In these mice Src phosphorylation on Y529 is increased (293, 294). Further, overexpression of PTPα in fibroblasts increases Src activity and promotes neoplastic transformation. indicating that PTPα activity is directed against Src (162). In this context, the study showed that phosphorylation on Y789 of PTPα is required to conduct PTPα action. Overexpression studies with PTPα mutants provide evidence that Y789 phosphorylation is required for selective Src Y529 dephosphorylation and subsequent FAK activation (204, 295). The Grb2 SH2 domain was reported to bind to Y789 of PTPα with higher affinity than the Src SH2 domain. Thus, these results suggest that Grb2 binding to PTPα competes with Src binding and activation in cells (203). Zheng and colleagues proposed a "phospho-displacement mechanism" describing Src activation by PTPα (figure 13). Notably, this model became the most common model for Src activation by PTPs, although it still remains controversial and still gives rise to a number of debates (296). Based on this model, Grb2 is released from Y789 of PTPα during mitosis. The phosphorylated Y789 is now accessible and promotes the dissociation of Src Y529 from the intramolecular binding with the Src SH2 domain (figure 13). The opening of Src structure facilitates its interaction with the PTPα D1 catalytic domain and resulting in the dephosphorylation of Y529 (204). In contrast, another study recently proposed an alternative Src activation model for PTPα, challenging the role of phosphorylated Y789 in Src activation. The authors elucidated the role of serine phosphorylation known to increase PTPα activity during mitosis. Previous studies showed that PTPα activation is supported by serine phosphorylation (297, 298). However, Vacaru and colleagues demonstrated that dephosphorylation of Ser204 enhanced PTPα affinity for Src, leading to dephosphorylation of tyrosine 529 and 418 of Src and a moderate Src activation in mitosis (206).

Interestingly, PTPα WT can also dephosphorylate Src tyrosine 418 in vitro and in vivo suggesting that PTPα regulation of Src activity is complex and can be activating or inactivating dependent on the cell context and external factors.

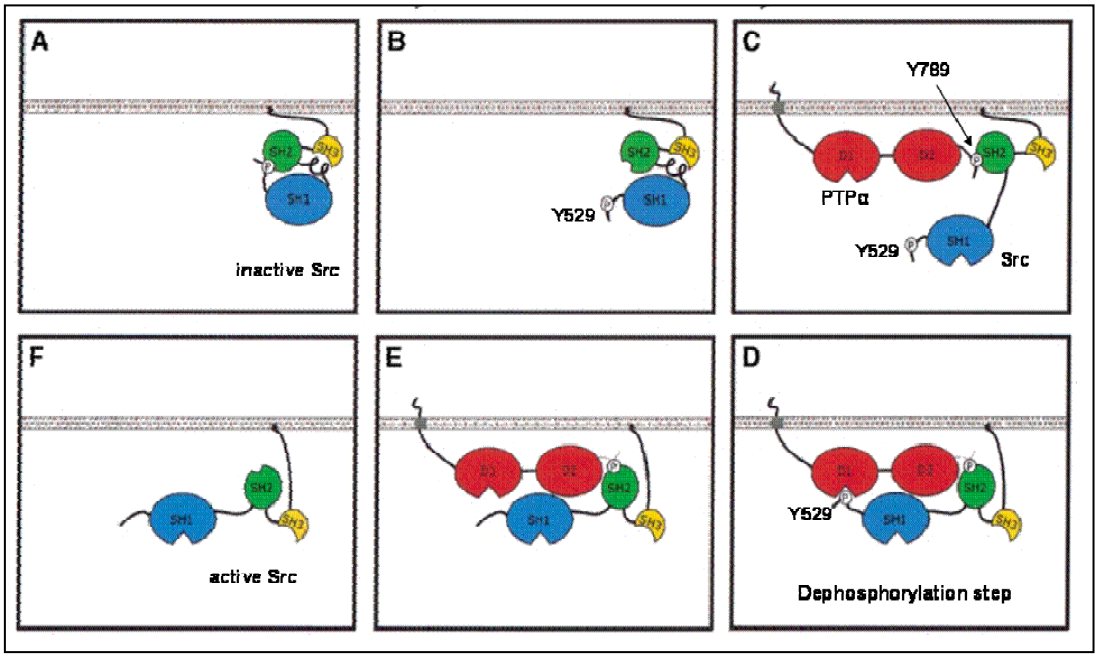

Figure 13: Mechanism of Src activation via the phospho-displacement model.

This model was described by Zheng and colleagues in 2000. A) In basal conditions Src is inactive and in a closed conformation. This conformation keeps Src locked through the intramolecular interaction of Y529 with the SH2 domain. B) Some particular conditions can induce the transient detachment of Src Y529 from the SH2 domain without its dephosphorylation. C-E) This intermediate state allows that Y789 of PTPα can bind to the SH2 domain of Src. Src can adopt a favourable conformation to be dephosphorylated by the catalytic D1 domain PTPα. F) Src Y529 is dephosphorylated and the phosphorylation of Y418 in the activation loop increases, leading to Src activation. Adapted from (204).

1.7.2.2 PTPε

PTPε is a member of the same RPTP family as PTPα and substrate-trapping mutants of PTPε provide evidence that Src is a substrate of PTPε as it was shown for PTPα. Two major isoforms of PTPε exist and both forms are able to activate Src (299-302). Studies in PTPε $^{-/-}$ mice confirmed that PTPε is an activator of Src in vivo and a collaborator of Neu in mammary tumour cells, participating in the maintenance of the Neu-induced transformed phenotype by Src activation (303). In the same context, phosphorylation of Y695 was reported to direct specificity of the receptor-type PTPε toward Src in Neu-expressing mammary tumour cells. Y695 is located in the C-terminal domain of PTPε and seems to be exclusively implicated in the dephosphorylation of Src but not in that of other substrates (299). Another study of the same group showed that in osteoclasts, the non-receptor form of PTPε (cyt-PTPε) is phosphorylated in a Src-dependent manner on Y638 and participates in the appropriate activation of Src downstream of integrins. Phosphorylated Y638 allows Src binding to PTPε, leading to Src Y529 dephosphorylation and its activation (300). Additional studies also demonstrated that PTPε can associate with and activate Fyn and Yes through the dephosphorylation of Y529 (304).

1.7.2.3 CD45

SFK and CD45 are important players of signal transmission downstream of immune receptors in B and T cells, but also in macrophages (305). CD45 was reported to catalyze dephosphorylation of tyrosine 529 and 418 of SFK, although the activating Y418 is dephosphorylated to a smaller extent than Y529, proposing a role of CD45 in SFK activation. Most studies performed in T cells showed that CD45 is essential for the dephosphorylation of Y529 of SFK, which primes the kinase to phosphorylate ITAM-motifs in the TCR to transmit cellular signals (306, 307). In B cells and

macrophages, CD45 deficiency was less severe than in T cells, providing evidence that CD45 is not the unique SFK activator in these cells (308, 309). CD45 was proposed to act concomitantly with DEP-1 (CD148) to activate Src in B cells. Double knockout studies of CD45 and CD148 in mice generated developmental defects in B cells and the myeloid lineage and also altered immune receptor signalling and hyperphosphorylated SFK, proposing overlapping roles for both PTPs in SFK activation in these cells (272).

1.7.2.3 SHP-1 and SHP-2

SHP-1 and SHP-2 are cytosolic PTPs containing several SH2 domains and are thought to contribute to growth receptor signalling pathways via SH2 domain interaction. Both PTPs regulate Src activity by influencing Y529 phosphorylation. In the case of SHP-1, it was shown to associate with Src and directly dephosphorylate Y529 (310). Interestingly, Src was also shown to catalyze SHP-1 tyrosine phosphorylation in the C-terminal tail, leading to SHP-1 activation (311).

SHP-2 regulates Src activity in an indirect manner via the regulation of Csk localisation. PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains), VE-cadherin and Paxillin are sites/proteins where Csk is recruited to the membrane via its SH2 domain (261, 312, 313). At these sites Csk regulates Src activity through the phosphorylation of Src Y529, leading to Src inactivation. SHP-2 can be co-recruited to these sites and dephosphorylates the Csk binding sites on these proteins, allowing the release of Csk. The dissociation of Csk from these binding sites leads to the decrease of Src Y529 phosphorylation, allowing Src activation. Thus, SHP-2 indirectly promotes Y418 phosphorylation and Src activation through the regulation of Csk access to Src in response to growth factor stimulation and integrin signalling.

1.7.2.4 PTP1B

PTP1B is an abundant cytosolic PTP with a major role in the regulation of proliferative and metabolic signals. Tyrosine 529 of SFK is a target of PTP1B downstream of integrin and insulin signalling as well as in several human breast cancer cell lines (314, 315). Moreover, the activation with SFK can also be mediated by distinct interaction domains such as two proline-rich regions in the C-terminal tail or a tandem tyrosine residue in the N-terminal of PTP1B (316). In breast cancer cells, activation of ErbB2 increases PTP1B expression, resulting in higher Src activation and the induction of a Src-dependent transformed phenotype in these cells (317).

1.7.2.5 DEP-1

DEP-1 is ubiquitously expressed and several studies provide evidence that DEP-1 can regulate Src activity. In malignant rat thyroid cells, overexpressed DEP-1 dephosphorylates Src on tyrosine 529 leading to its activation (318). Another study showed that DEP-1 promotes adhesion-dependant Src activation in PDGF-stimulated PAE and NIH3T3 cells (319). Moreover, somatostatin (SST) was shown to activate DEP-1 in CHO cells stably expressing SST1 receptor (SST1R). In these cells, DEP-1 was reported to interact with Src and to be activated by the JAK2/SHP2/Src pathway following somatostatin stimulation. This study did not find evidence that Src can be dephosphorylated by DEP-1 in response to SST (212). Thus, several studies provide hints that DEP-1 can interact with and activate Src in various cell types. However, it is not known if Src activity is affected in DEP-1 knockout models or the DEP-1 knockin model (265, 272, 320). Our laboratory demonstrated recently that Src activity is increased in a DEP-1-dependent manner in VEGF-stimulated EC, leading to increased cell survival (figure 14) (160). However, the mechanism of Src

activation via DEP-1 and the consequences on other endothelial cell functions in response to VEGF remain poorly defined.

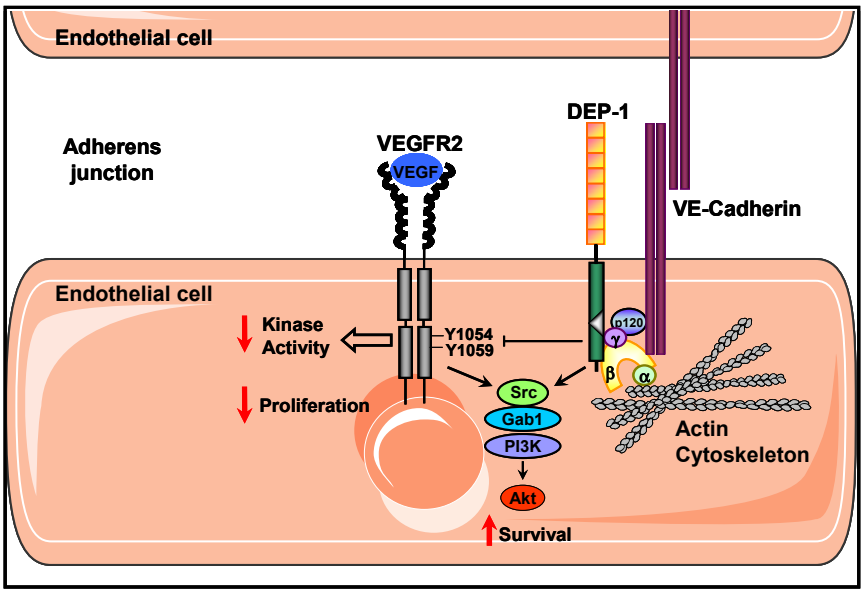

Figure 14: Implication of DEP-1 in the regulation of VEGFR2 phosphorylation and VEGF-dependent downstream signalling.

Upon VEGF stimulation, DEP-1 can target tyrosine 1054/1059 of VEGFR2 to reduce the general VEGFR2 phosphorylation at adherens junctions. Common signalling pathways including ERK1/2 and p38 are downregulated with the exception of the Src/Gab1/AKT pathway, which is induced under these conditions, leading to increased survival of EC. Adapted from (149, 160).

1.8 Biological functions of Src in endothelial cells

Src has a central role in cell signalling, resulting in the mediation of basal EC functions such as cell migration, survival and cell division (321-325). Adherens junctions are composed of VE-cadherin, which is linked to p120 catenin, plakoglobin and β-catenin via its cytoplasmic tail (326). In response to VEGF, tyrosine phosphorylation of adherens junction proteins is reported to increase (135). Src is

proposed to be responsible for the phosphorylation of VE-cadherin since VEGF-induced VE-cadherin phosphorylation is absent in $Src^{-/-}$ mice (65). Src induces VE-cadherin phosphorylation via two mechanisms in response to VEGF. On one hand, Src phosphorylates Vav2, resulting in the activation of Rac and PAK, which in turn phosphorylates serine 665 of VE-cadherin (137). On the other hand, Src mediates the direct phosphorylation of VE-cadherin at Y685 (327). Both Src-dependent phosphorylation processes induce the internalization of VE-cadherin in response to VEGF, resulting in the loss of junctional integrity. Moreover, Src also phosphorylates β-catenin on Y654, resulting in its release from VE-cadherin (328, 329). Following phosphorylation, the catenin-cadherin complex is abrogated and its adhesive function is lost, resulting in the loosening of cell-cell junctions (136, 330, 331). Decreased adhesive function of cell-cell contacts induces cell migration and invasion. Moreover, the remodelling of cell junctions induces vascular permeability through intercellular gap formation, allowing the passage of molecules and fluids into the interstitial space (129, 135, 323, 332). The entirety of induced changes of endothelial cell contacts upon VEGF stimulation allows the sprouting of endothelial cells to form a new capillary.

VEGF also induces vascular permeability involving the eNOS pathway as shown in an $eNOS^{-/-}$ mouse model (140). Interestingly, it was reported that Src induces the activation of the eNOS pathway upon VEGF stimulation. The study showed that in basal conditions, Csk is bound to VE-cadherin and negatively regulates the activity of VE-cadherin-associated Src. Upon VEGF stimulation, SHP-2 is co-recruited to VE-Cadherin, dephosphorylating the Csk binding site Y685. This results in the release of Csk into the cytosol and Src activation. The activated Src/VE-Cadherin/SHP-2 complex induces the activation of the eNOS/AKT pathway, resulting in the disruption of cell contacts (261).

Besides paracellular transport, Src also plays a major role in transcellular transport. This second transport mechanism permits the active passage of macromolecules into the interstitial space through the Src-mediated formation of

calveolae and their detachment as endocytic vesicles (331, 333). Both cellular transport mechanisms are implicated in physiological as well as pathological processes including inflammation and extravasation of tumour cells from the vascular space (334, 335). Indeed, the loss of functional cell-cell junctions has been associated with increased invasion and metastases in cancer (336). Src-mediated permeability allows cancer cells to invade and to metastasize into distant organs. Consistently, Src inhibition was shown to block metastasis formation (337-339).

1.9 Biological functions of Src in cancer cells

Based on the central role of Src in the regulation of multiple signalling networks, its activation has to be tightly regulated in a time- and space-dependent manner. Inappropriate Src activation is implicated in various pathologies such as cancer. Thus, high levels of Src expression and activity are observed in most human cancer types including colorectal, ovarian, pancreatic and breast cancer (340-343). However, Src mutations are rarely found in cancers, indicating that its activity is altered by upstream events including overexpression and altered activity of RTKs and PTPs. Consistent with these observations, v-Src, which lacks the inhibitory Y529, is a strong tumour promoter and it is the first oncogene identified as well as one of the most studied. Truncation of its C-terminal tail, which encompasses the inhibitory tyrosine Y529, results in a constitutively active form. In cancer cells, the constitutively active Src increases proliferation and cell survival to promote tumour mass formation. On the other hand, it also promotes the remodelling of the Actin cytoskeleton and cell-cell and/or cell-matrix adhesion, thereby facilitating cell motility and invasion (344). Activation of downstream targets of Src associated with higher cell motility including p190 RHOGAP leads to the loss of Actin organization and focal adhesion disassembly, resulting in cell detachment from the ECM (345-348). Cancer cell invasion is an essential step to form distant metastases. The

"mesenchymal-type" invasion combines migration and ECM breakdown by cancer cells and involves Src activity to trigger expression of matrix-degrading proteases including MMP2 and MMP9 (349). Src was also reported to inhibit endocytosis of MT1-MMP, leading to its higher expression at the cell surface and hence in higher activation of its substrate MMP2 (350). Epithelial-to-Mesenchymal Transition (EMT) is a common feature of epithelial cancer cells and a dynamic process that endows cells with a higher capacity to migrate. It promotes the migration of single cells, which is a distinct feature of mesenchymal cells (351-353). In addition, Src can induce the phosphorylation of the E-Cadherin-β-catenin complex, leading to the disruption of adhesive cell contacts and the promotion of invasion and the EMT phenotype in cancer cells (339, 354-356). Phosphorylated β-catenin translocates to the nucleus to induce transcription of genes whose products such as proteases and vimentin are involved the promotion of EMT, invasion and tumour progression (354, 357). Src-mediating degradation of the Rac activator Tiam1, which is implicated in the maintenance of cell-cell contacts, allows enhanced motility and invasiveness (358). Thus, Src will favour increased migration and invasion of tumour cells to promote their dissemination and the formation of metastases. Consistent with these data, Src is involved in the formation of metastases to distant organs in breast cancer models, especially to the bone and the lung (338, 359, 360). In conclusion, in both endothelial and cancer cells Src-mediated loosening and disruption of cell-cell contacts will contribute to tumour progression and metastasis formation.

1.9.1 The role of Src in breast cancer

In breast cancer, numerous genes are deregulated by heterogeneous gene copy numbers, expression abnormalities and mutations. Genome-wide analysis of gene expression in breast cancer cell lines and primary tumours led to their classification in various subtypes (361, 362). Common subsets were defined as basal-like, luminal-

like A and B, Her2-enriched and normal-breast-like. The basal-like type is commonly characterized by the lack of hormone receptors for estrogen and progesterone but also the lack of Her2 and E-cadherin expression. In contrast, the expression of EGFR, basal cytokeratins, and Vimentin is amplified (363, 364). This subtype is a poor prognosis group based on its in generally more invasive character and its higher aggressiveness resulting in the formation of distant metastases (362, 365, 366). On the other hand the luminal-like group displays expression of ER and Her2 along with expression of epithelial markers such as cytokeratins and E-cadherin (363, 367). According to its molecular pattern, luminal-like breast cancer is in general less aggressive, and patients have a better outcome than those with a basal-like breast cancer (368). Based on heterogeneous expression patterns, subsets can also encompass several subset entities, as it is the case for triple-negative breast cancer. This subtype is negative for hormone receptors expression and Her2 expression but shows various molecular characteristics of basal-like as well as normal-like breast cancer (369).

Src kinase activity and expression is increased in breast cancer tissue compared to normal breast tissue (370). Src is a mediator of many downstream effects of RTK and crosstalk between EGFR family and Src was shown in breast cancer specimens (371). Notably, Src transfection was shown to increase EGF-mediated oncogenesis (372). For instance, SFK activity is essential for MAPK activation following growth factor stimulation in various breast cancer cell lines, and in consequence, for the induction of breast cancer cell proliferation (373). Furthermore, DNA synthesis in response to EGF was reported to be dependent on a functional Src kinase domain (374). Another study demonstrated that Src is required for cell motility and anchorage-independent growth in response to activation of the Her2-Her3 complex (375). Since bone is the preferred site for the formation of metastases in breast cancer, it is interesting that Src is known as an essential regulator of osteoclast function (359). These results demonstrated a role for Src in the formation of metastases to bone. In addition, increased Src expression was correlated

with the upregulation of mesenchymal markers such as Vimentin and N-Cadherin and with a higher invasive, capacity suggesting its implication in the EMT of breast cancer (355, 360). Altogether, these studies demonstrated an implication of Src in breast cancer progression, providing the rationale to target Src or to modulate its activity for breast cancer treatment. Some Src inhibitors such as dasatinib are currently in clinical trials for patients with solid tumours such as breast cancer (376).

1.10 The role of PTPs in cancer

Given that the Src activity is regulated by PTPs and that Src in turn regulates multiple signalling pathways in cancer cells, the role of PTPs in cancer recently became a subject of interest in cancer research. High tyrosine phosphorylation levels are observed in breast cancer tissue compared to normal mammary tissue due to elevated tyrosine kinase activity, suggesting that the balance between RTKs signalling and PTP-mediated dephosphorylation is altered (377, 378).

1.10.1 Tumour suppressing PTPs

Since PTPs have antagonistic effects on RTKs and their dependent signalling, they have initially been considered as tumour suppressors. Often, PTPs are mutated and/or inactivated in cancer. Epigenetic alterations such as methylation are the most common mechanism for gene inactivation resulting in a global loss of gene expression compared to normal tissue (379). Further, as reported for other tumour suppressor genes, deletion, mutations or loss of heterozygosity (LOH) are common genetic alterations of PTP genes (380). The most frequent gene alterations of RPTPs in cancer are found in the extracellular domain. Structure-prediction studies proposed that a number of mutations in the extracellular domain of RPTPs could alter their

organization and change their interaction with other proteins. Further, point mutations in the catalytic domain predicted to reduce its catalytic activity were reported for PTPRT in colorectal cancer (381). Inactivating mutations in PTPs commonly occur together with activating mutations in RTK, counter-steering the general balance of RTK- and PTP-dependent signalling in cancer.

1.10.1.1 GLEPP1/PTPRO

PTPRO was reported to be inactivated by promoter methylation in human lung cancer. Moreover, in A549 lung cancer cells, overexpressed PTPRO reduced anchorage-independent growth, proliferation and the resistance to apoptosis (382). Furthermore, inhibiting methylation of PTPRO gene in leukaemia results in higher phosphorylation of BCR/Abl, increasing its transformation potential (383). In addition, promoter methylation of GLEPP1/PTPRO leading to the suppression of PTPRO expression is also seen in the breast cancer cell lines MCF-7 and MDA-MB 231, and in many primary breast cancer tumours (384). The inhibition of PTPRO expression was proposed to be estrogen-dependent, consistent with results showing that PTPRO expression increases after treatment with the estrogen receptor antagonist tamoxifen. Inverse experiments showed that PTPRO overexpression increased tamoxifen sensitivity in MCF-7 cells (385). Thus, these data support a role for PTPRO as a tumour suppressor.

1.10.1.2 SHP-1

Epigenetic alterations such as hypermethylation of the SHP-1 promoter lead to its inactivation in cancer. This alteration was first described in T cell lymphomas and results showed that the treatment with a demethylating agent restored SHP-1

expression (386, 387). Re-expression of SHP-1 decreased the phosphorylation of JAK3 or STAT3 in this model, antagonizing the JAK3/STAT3-induced cell survival (388, 389). Some studies demonstrated that SHP-1 mediates somatostatin-induced apoptosis by increasing cellular acidification and contributes to the antiproliferative effect of SST2 in MCF-7 and T47D cells, supporting its negative role in cancer (390, 391).

1.10.1.3 DEP-1

DEP-1 was demonstrated to be a tumour suppressor in many cancers. In various breast cancer cell lines, DEP-1 was shown to mediate growth inhibition in vitro (392). Interestingly, DEP-1 expression was associated with higher differentiation of several types of cancer cells, suggesting an inhibitory role for DEP-1 in cell transformation (392, 393). In agreement with these studies, DEP-1 expression was shown to suppress the malignant phenotype of transformed rat thyroid cells (394). Moreover, the functional PTPRJ gene was associated with the colon cancer susceptibility locus Scc1 in mice. Originally this locus was defined by its segregation with colon cancer susceptibility after crossing cancer-resistant with cancer-susceptible mouse strains. Notably, sequence differences in DEP-1 between the cancer-resistant and the cancer-susceptible strain support the notion that certain DEP-1 variants drive cancer susceptibility due to inactivating mutations (395-397). Further analysis of the PTPRJ gene status in human samples identified that LOH occurs in PTPRJ in colorectal cancer as well as breast, lung, thyroid cancer and meningiomas (figure 15 A-B) (395, 398). Studies in thyroid carcinoma, colon and breast cancer patients demonstrated that often the preferential loss of one allele occurs during cancer growth, indicating that the two DEP-1 alleles have different activities; the one remaining is potentially less active (395, 396, 399). Moreover, single nucleotide polymorphisms (SNPs) found in the DEP-1 extracellular domain were shown to

influence the susceptibility to a wide range of cancer types including colorectal, lung, head and neck cancer (400). As in mice, SNPs generate different allelic variants of PTPRJ in humans, which differ in their extracellular domain (figure 15 C) (395). Hence, these results suggest that DEP-1 could be less active in several cancer types due to the preferential loss of the more active allele and inactivating mutations in the remaining allele.

Further examples strengthen the tumour suppressive role of DEP-1 in different cancer types. For instance, EGFR is known to drive several types of malignancy such as breast, head and neck as well as lung cancer (401). Thus, it is remarkable that DEP-1 inhibits EGFR activation at the cell surface along with inhibition of the active receptor transport to endosomes, supporting the tumour-suppressive function of DEP-1 consistent with former studies (213). Also consistent with these studies, DEP-1 expression was associated with inhibition of proliferation and motility in meningiomas and a colon cancer cell line (398, 402). Cytoskeletal rearrangements were induced by DEP-1 in colon cancer cells, leading to inhibited migration in comparison to DEP-1-negative colon cancer cells (402). Recently, Flt-3 transformation of lymphoma cells in acute myeloid leukaemia (AML) was shown to be accompanied by the inactivation of DEP-1, suggesting that DEP-1 activity is required to suppress cell transformation (223).

Some studies also provided initial evidence that DEP-1 could also have other functions in cancer in addition to its tumour-suppressing features. Surprisingly, DEP-1$^{-/-}$ mice grow and develop normally and show no spontaneous development of tumours, suggesting that additional alterations are necessary for cancer development (320). Recently, a missense mutation in the non-catalytic domain of DEP-1 (K1017N) was shown to be enriched in primary breast tumours. Additionally, this DEP-1 variant was also found amongst the most highly enriched mutations in brain metastases (403). Thus, these results suggest that some DEP-1 mutations could be favourable for cancer progression. Interestingly, another study showed that expression of the DEP-1 K1016A mutant impaired the interaction between the known

DEP-1 substrate ERK1/2 and DEP-1. This mutant is unable to dephosphorylate ERK1/2, resulting in continued cell proliferation (404). It is possible that the DEP-1 K1017N mutation found in brain metastases of breast cancer has similar functions on ERK1/2 as the DEP-1 K1016A mutant (380).

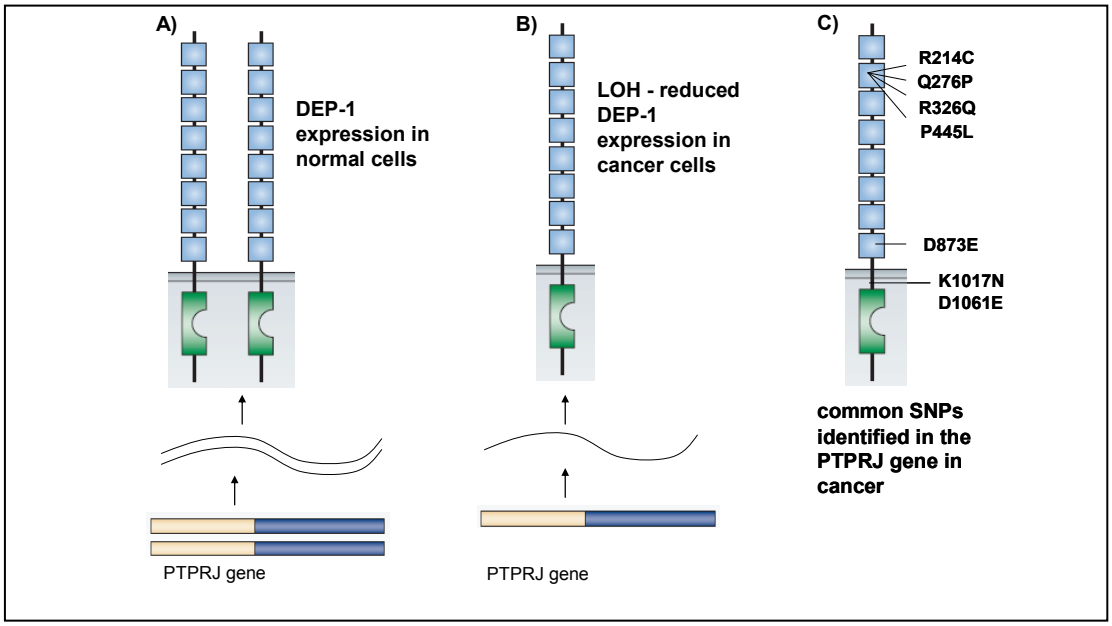

Figure 15: Principal mechanisms of DEP-1 modification in cancer.

A) DEP-1 is ubiquitous expressed in normal cells. B) Ruivenkamp et al., 2003 found loss of hetereozygosity in the PTPRJ gene, which encodes for DEP-1, in samples of different cancer types. LOH is a common gene modification in cancer and results in the loss of one allele while the other less active allele remains. C) Five single nucleotide polymorphisms in the fibronectin motifs of the DEP-1 extracellular domain were identified in colorectal cancer samples. The DEP-1 K1017N mutation was found enriched in breast metastases. Adapted from (380, 396, 405).

1.10.2 Tumour promoting PTPs

Oncogene activation can be induced by certain gene alterations such as chromosome translocation, activating point mutations (gain-of-function mutations) and gene amplifications. These activating alterations are frequently found in genes encoding for PTPs suggesting a tumour-promoting role for these PTPs in cancer.

1.10.2.1 PTPα

PTPα was believed to be a potential oncogenic PTP due to its ability to activate Src. Its positive effect on cell signalling allows PTPα to transform mouse fibroblasts, suggesting that PTPα can act as an oncogene itself or enhance tumorigenicity in collaboration with other factors (162, 406). However, Src-activating PTPα mutants are expressed in late stage colon and breast carcinoma (406). Depletion of PTPα results in decreased Src activity in mouse brain, mouse fibroblasts and several human cancer cell lines including breast and colon (293, 294, 407). Moreover, Src activity was inhibited in PTPα-depleted ER-negative breast cancer cell lines concomitantly with suppressed anchorage-independent growth and reduced cell survival, suggesting a tumour-promoting role for PTPα in these cells. In contrast, PTPα depletion did not inhibit Src activity in ER-positive breast cancer cell lines and its expression was correlated with low tumour grade and ER expression (407, 408). Together, these results suggest that PTPα can promote tumour progression depending on the ER status in breast cancer (407). Recently, it was shown that two distinct isoforms of PTPα exist, which both can activate Src, but differ in their capacity to transform cells due to a different extracellular domain, adding an interesting aspect to PTPα regulation (409).

1.10.2 .2 PTPε

Expression of PTPε was shown to promote mammary hyperplasia in transgenic mice, and these mice developed sporadic mammary tumours more frequently than WT mice (410). PTPε can activate Src and promotes the transformed phenotype of Neu-induced mammary tumour cells. Its depletion decreases cell proliferation and cells appear morphologically less transformed (303). This PTPε-mediated Src activation is essential to maintain the transformed phenotype of Neu-induced mammary tumour cells, demonstrating the tumour promoting role of PTPε in this model (299). Recently, it was shown that the expression of PTPε promotes anchorage-independent growth and increases survival in human breast cancer cell lines, supporting the notion that PTPε can act as a tumour promoter (411).

1.10.2.3 SHP-2

Mutations due to single-amino acid changes are found in the catalytic and the SH2 domain of SHP-2 and increase its basal activity by preventing the inhibitory conformation of SHP-2. Thus, SHP-2 is defined as a proto-oncogene activating oncogenic tyrosine kinases in several cancers including leukemia (412-414). The activating mutations were detected in half of the cases of Noonan syndrome and in several human cancers including leukaemia, lung and colon cancer, melanoma and neuroblastoma (415-417). Further mutated variants encompass modified substrate selectivity. For instance, the activation of Ras-ERK is the principal effect of SHP-2 mutants leading to transformation of cells (405, 412).

1.10.3 Dual role of certain PTPs in cancer – PTP1B

The role of PTP1B in cancer is contentious. A number of studies showed a tumour-promoting role for PTP1B, suggesting that PTP1B can act as a proto-oncogene. In

contrast, it can also act as a tumour suppressor depending on the cancer type and/or the mutations of other genes involved in cancer development (418).

Although PTP1B deficiency promotes oncogenic signalling due to the absent negative regulation of various growth receptors, PTP1B knockout mice lack spontaneous tumour development. Interestingly, PTP1B expression is elevated in most breast cancer specimens and positively correlates with ErbB2 expression. Consistent with this observation, PTP1B was shown to be a positive regulator of ErbB2 signalling, promoting the activation of the ERK- and PI3K/AKT-pathway. These events enhance mammary tumorigenesis and malignancy in breast cancer (419-421). Similar results were observed in ovarian cancer, suggesting that ErbB2 and PTP1B could cooperate in different cancer types to promote tumorigenesis (422, 423). Interestingly, analysis of ErbB2 mutant mice that are reported to develop spontaneously mammary carcinomas, were crossed with $PTP1B^{-/-}$ mice (424). The PTP1B deficiency in these mice delays tumour progression through the downregulation of the ERK- and PI3K/AKT-pathway and protects from lung metastasis formation (420, 421). Moreover, the activation of the Ras pathway was shown to be decreased in fibroblasts isolated from PTP1B knockout mice, indicating that PTP1B has a pro-oncogenic role in part due to the enhanced Ras signalling (425). In agreement with PTP1B knockout studies, PTP1B overexpression increases Src activation in colon cancer cells, further suggesting an activating role of PTP1B in cancer cell growth and invasion (315, 426). Consistent with this, increased PTP1B activity was reported to promote Src activation and invadopodia formation in breast cancer cells (427).

In contrast to this positive implication of PTP1B in cancer, anti-tumorigenic functions of PTP1B are also reported. PTP1B overexpression was shown to decrease v-Src and Crk-induced ERK activation (428). Consistent with its tumour-suppressive role, various studies demonstrated that PTP1B promotes apoptosis through different mechanisms including downregulation of pro-invasive signalling, ER-stress induction or the increase of caspase 8/9 activity (429-432). Notably, PTP1B is also implicated

in the regulation of cell junctional integrity, reducing cell motility. It was shown to associate with N-cadherin to dephosphorylate β-catenin, promoting the complex formation between N-cadherin and β-catenin to strengthen cell-cell contacts (433, 434). Genetic ablation of PTP1B in a p53 knockout mouse model revealed that PTP1B indirectly delays lymphomagenesis via the promotion of B-cell differentiation in these mice (435).

Currently, a body of evidence suggests for various PTPs that the same PTP may behave as a tumour promoter as well as a tumour suppressor in cancer. Genetic and/or epigenetic alterations are suggested to act on PTP genes to modify the protein function depending on the cell context, resulting in this dual role in cancer.

1.11 Previous work and objectives of this thesis

DEP-1 (Density-enhanced phosphatase 1) was shown to be implicated in vessel remodelling and branching during development, suggesting an essential role in the regulation of angiogenesis. Its expression increases with cell density and was shown to correlate with the dephosphorylation of VEGFR2 in confluent endothelial cells. However, the contribution of DEP-1 in the regulation of VEGFR2 and VEGF-dependent signalling pathways and biological responses remained ill-defined. Previous work in our laboratory showed that DEP-1 negatively regulates VEGFR2 activity by the dephosphorylation of tyrosines 1054 and 1059 in the kinase activation loop of VEGFR2. In consequence, the global phosphorylation of VEGFR2 is decreased and the activation of most signalling pathways downstream of VEGFR2 is impaired. Consistently, DEP-1 depletion results in the global increase of VEGFR2 phosphorylation and in the increased activation of major downstream pathways including the mitotic PLCγ-ERK1/2 pathway and p38. In contrast, the phosphorylation of Src on its inhibitory tyrosine 529 is increased in these cells

suggesting that DEP-1 promotes the activation of Src. Despite the negative role of DEP-1 on most VEGF-dependent signalling pathway, our study revealed that DEP-1 induces the Src-Gab1-AKT pathway and promotes endothelial cell survival. However, the specific molecular mechanism implicated in DEP-1-mediated Src activation as well as the understanding of the role of DEP-1 in the regulation of the VEGF-dependent angiogenic program remained ill-defined.

In the course of our studies, we observed that DEP-1 is phosphorylated on tyrosine and since it is known that the tyrosine phosphorylation of PTPs can direct them towards specific substrates and/or regulate PTP activity, we were interested to identify major phosphorylation sites in DEP-1 and to elucidate the implication of these sites on DEP-1 function in endothelial cells. In detail we wanted to determine:

- that DEP-1 is phosphorylated in endothelial cells in response to VEGF on the identified tyrosines
- that the phosphorylated tyrosines of DEP-1 are implicated in Src binding and activation
- that DEP-1 and its tyrosine phosphorylation are implicated in the regulation of endothelial cell functions such as permeability, capillary formation and invasion
- the impact of DEP-1 expression levels on Src activation and endothelial cell functions

In this first study, we showed that Y1311 and Y1320 are the major tyrosine residues which are phosphorylated in the C-terminal tail of DEP-1. These residues are essential for Src activation and the regulation of endothelial cell functions including permeability, invasion and capillary formation. Subsequently, we identified also a threonine residue proximal to Y1320 which displays a potential phosphorylation site.

The aim of this second study was to define the role of T1318 on DEP-1 tyrosine phosphorylation and consequently in Src activation and endothelial cell functions. The results are presented in chapter III:

- Confirmation of DEP-1 phosphorylation on threonine
- Evaluation of DEP-1 T1318 phosphorylation in endothelial cells
- Impact of DEP-1 T1318 phosphorylation on Y1311 and Y1320 phosphorylation
- Role of DEP-1 T1318 phosphorylation on Src binding and Src activation
- Consequence of DEP-1 T1318 phosphorylation on the phosphorylation of VE-cadherin and endothelial cell permeability
- Identification of the kinase responsible for DEP-1 T1318 phosphorylation

In this second study, we demonstrated that DEP-1 is constitutively phosphorylated on T1318 in endothelial cells. Upon VEGF stimulation, T1318 phosphorylation is decreased concomitantly with induced DEP-1 phosphorylation on Y1311 and Y1320. Mutation of DEP-1 T1318 impairs phosphorylation of Y1311 and Y1320, Src activation and endothelial cell permeability. First hints provide evidence that DEP-1 T1318 is phosphorylated by CK2 in endothelial cells.

Src is known as tumor oncogene, promoting cancer progression via the induction of cell transformation, pro-invasive cell signalling and cell functions. Since DEP-1 has a major role in Src activation in endothelial cells, we were interested in understanding the role of DEP-1 in Src regulation in breast cancer cells. The results of this third study are presented in chapter IV:

- Evaluation of DEP-1 expression in a panel of basal-like and luminal-like breast cancer cell lines
- Implication of DEP-1 in Src regulation in this panel of breast cancer cell lines
- Effect of DEP-1 on cell signalling events (induction of pro-invasive pathways) in basal-like breast cancer cells
- Determination of the role of DEP-1 on biological cell functions in basal-like versus luminal-like breast cancer cell lines
- Confirmation of our model in a bone metastatic subpopulation of MDA-MB 231 (variant 1833)
- Evaluation of DEP-1 expression in human breast cancer specimen (TMA) and its impact on the clinical outcome of breast cancer patients

In this study we showed that in basal-like breast cancer cell lines DEP-1 expression is elevated in contrast to luminal-like breast cancer cell lines. DEP-1 activates Src and Src-dependent pro-invasive signalling in basal-like breast cancer cell lines. Consistently, DEP-1 is required for the proper localisation of proteins implicated in the mediation of cell motility and in the promotion of migration and invasion in basal-like breast cancer cell lines. Importantly, DEP-1 expression is correlated with reduced overall survival of patients in human breast cancer.

This thesis contains the following articles:

1. **Tyrosine phosphorylation of DEP-1/CD148 as a mechanism controlling Src kinase activation, endothelial cell permeability, invasion and capillary formation.**
   16 Aug 2012 (Epub ahead of print)

2. **DEP-1 Phosphorylation on threonine 1318 regulates its ability to promote Src activation, VE-cadherin phosphorylation and vascular permeability in endothelial cells.**
   In preparation

3. **Novel role for the protein tyrosine-phosphatase DEP-1/PTPRJ as a promoter of breast cancer cell migration and invasion.**
   Submitted for publication

CHAPTER II

Tyrosine phosphorylation of DEP-1/CD148 as a mechanism controlling Src kinase activation, endothelial cell permeability, invasion and capillary formation

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Author contribution

K.S. has done experiments for figure 1C and E, 2F, figure 3A, D in collaboration with L.L. and 3C, figure 4A and B, figure 6 and 7, supplementary figure 2C, D and supplementary figure 3.

C.C performed experiments for figure 1B and D, figure 2B and C.

S.L. performed experiments for figure 1F, 4C and D, figure 5, supplementary figure 1B.

L .L. performed experiments for figure 2A, figure 3B and E and supplementary figure 1A and 2B. Figure 3A and D was performed in collaboration with K.S.

Christine C. performed experiments for Supplemental figure 2.

N.T.N.T. generated myr-DEP-1 Y/F mutants for figure 2B and C.

J.G., J.H. and M.E. participated to discussions and contributed reagents and protocol.

I .R. designed the research project and analyzed the data and wrote the paper with the contributions of K.S. and C.C.

Abstract

DEP-1/CD148 is a receptor-like protein tyrosine phosphatase with anti-proliferative and tumor-suppressive functions. Interestingly, it also positively regulates Src family kinases in hematopoietic and endothelial cells, where we showed it promotes VE-cadherin-associated Src activation and endothelial cell survival upon VEGF stimulation. However, the molecular mechanism involved and its biological functions in endothelial cells remain ill-defined. We demonstrate here that DEP-1 is phosphorylated in a Src- and Fyn-dependent manner on Y1311 and Y1320, which bind the Src SH2 domain. This allows DEP-1-catalyzed dephosphorylation of Src inhibitory Y529 and favors the VEGF-induced phosphorylation of Src substrates VE-cadherin and Cortactin. Accordingly, RNAi-mediated knockdown of DEP-1 or expression of DEP-1 Y1311F/Y1320F impairs Src-dependent biological responses mediated by VEGF including permeability, invasion and branching capillary formation. In addition, our work further reveals that above a threshold expression level, DEP-1 can also dephosphorylate Src Y418 and attenuate downstream signaling and biological responses, consistent with the quiescent behavior of confluent endothelial cells that express the highest levels of endogenous DEP-1. Collectively, our findings identify the VEGF-dependent phosphorylation of DEP-1 as a novel mechanism controlling Src activation, and show this is essential for the proper regulation of permeability and the promotion of the angiogenic response.

Introduction

DEP-1/CD148 (also called PTPη or PTPRJ) is a receptor-like protein tyrosine phosphatase (PTP) expressed in several cell types including epithelial, endothelial and hematopoietic cells (436). It encompasses an extracellular domain containing eight fibronectin type III-like motifs, a transmembrane domain, a single intracellular catalytic domain, and a short C-terminal tail (234). Initial studies demonstrated that its expression increases with cell density and suggested a function in cell contact-mediated growth inhibition (234, 394). Overexpression of DEP-1 in cancer cells was also reported to inhibit their growth, while thyroid cell transformation was associated with its decreased expression, indicative of a role for DEP-1 as a tumor suppressor (392-394, 402, 437). DEP-1 was further identified as the gene associated with the mouse colon cancer susceptibility locus (*Scc1*), and was found to be frequently deleted and mutated in human cancers (438). These growth inhibitory functions of DEP-1 are consistent with the nature of some of its reported substrates, which include the PDGF, HGF (Met), and VEGF (VEGFR2) receptors as well as Src family kinases (SFKs), ERK1/2 and the p85 subunit of PI3K (149, 160, 173, 272, 274, 275, 318, 439, 440). DEP-1 also dephosphorylates proteins from the cell-cell junctional complexes including p120catenin, occludin and ZO-1, which might impact biological functions dependent on the loosening/strengthening of intercellular contacts (274, 275, 441).

VEGFR2 is a potent activator of the angiogenic response and is the main mediator of the mitogenic, chemotactic, permeability and survival effects of VEGF in normal and tumor-associated vessels (108). VE-cadherin adhesion complexes are important sites of VEGF-dependent signaling in confluent cells, as activated VEGFR2 associates to these complexes to mediate AKT activation and cell survival (269). DEP-1 was shown to co-localize to these sites and to attenuate the phosphorylation of VEGFR2, resulting in the impaired activation of ERK1/2 and the

contact-inhibition of endothelial cell proliferation (149, 442). However, DEP-1 was also reported to positively regulate VE-cadherin-associated Src and AKT and to promote VEGF-dependent endothelial cell survival (160). Consistent with these distinct in vitro functions, inactivation of DEP-1 in mice via the swapping of its catalytic domain and C-terminal tail with GFP revealed both positive and negative regulatory effects in vascular development, and resulted in defective vessel remodeling and branching in addition to increased endothelial cell proliferation (265).

SFKs promote cell survival, invasion, remodeling of cell-cell junctions and permeability which are essential cellular functions that contribute to vessel sprouting and neovascularization (18, 114, 158, 443, 444). In their inactive state, SFKs adopt a closed conformation where the SH2 domain is linked to the phosphorylated C-terminal tyrosine residue (Y529 in human Src). To become active, the SH2 domain must engage with higher affinity binding sites on other molecules to release the Src C-terminal tyrosine residue and allow its dephosphorylation by PTPs (phospho-displacement mechanism). This then induces conformational changes that result in trans-phosphorylation of the catalytic tyrosine residue (Y418 in human Src) and SFK activation (445). DEP-1 has been shown to dephosphorylate Src Y529 and Y418 in vitro, but to preferentially target Y529 and activate Src when overexpressed in thyroid tumor cells (318). Conversely, the silencing of DEP-1 in endothelial cells results in the increased phosphorylation of VE-cadherin-associated Src on Y529, consistent with the observed inhibition of its activity and decreased Y418 phosphorylation in response to VEGF (160). Recent genetic studies demonstrated that DEP-1/CD148 is also an important activator of SFKs in hematopoietic cells, and promotes immunoreceptor signaling, B cell and myeloid lineage development, phagocytosis, as well as platelet activation and thrombosis (272, 436, 446).

Despite their broad implication in the activation of SFKs, the molecular mechanism underlying the ability of most PTPs to activate Src remains incompletely understood. The phosphorylation of receptor-type PTPε and PTPα has been suggested to regulate their ability to activate Src (204, 206, 299). We show here that VEGF

induces the tyrosine phosphorylation on DEP-1 Y1311 and Y1320 in endothelial cells. This is essential for Src Y 529 dephosphorylation and the promotion of Src-dependent angiogenic responses (114, 129, 443). However, we also found that when expressed above a threshold level, such as that observed in confluent and quiescent cells, DEP-1 rather inhibits Src and downstream responses. This study therefore identifies for the first time regulatory events allowing the control of DEP-1-mediated Src activation and further defines the molecular mechanisms underlying the promotion of VEGF-dependent permeability and angiogenesis.

Methods

Antibodies and Reagents

PY99, Myc (clone 9E10), and DEP-1 (clone 143-41; for IP) antibodies were purchased from Santa Cruz Biotechnology. Antibodies detecting VEGFR2 p^{Y1059}, FAK, PLCγ, GST, and Src (mouse clone GD11 for IP and blotting) were obtained from Upstate/Millipore. DEP-1 goat (for blotting) and mouse (clone 143-41; for IP and blotting) antibodies were from R&D System. Phospho-specific antibodies against Src p^{Y418}, VE-cadherin p^{Y658}, FAK p^{Y861} and Alexa Fluor 488 goat anti-mouse antibody were from Invitrogen. Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgGs, VE-cadherin, Src (rabbit clone 36D10 for blotting), and Src p^{Y529} antibodies were purchased from Cell Signaling Technology, New England Biolabs. β-catenin antibody was from BD Transduction Laboratories. Anti-Fyn polyclonal antibody was provided by André Veillette, Institut de Recherches Cliniques de Montréal, Canada. VE-cadherin p^{S665} antibody was generated as described (137). Phospho-specific antibodies against DEP-1 Y1311 and Y1320 were generated by GenScript against the following peptides: CQKDSKVDLI[pY^{1311}]QNT and CKDSKVDLIYQNTTAMTI[pY^{1320}]EN, respectively. VEGF was obtained from the BRB Preclinical Repository of the NCI-Frederick Cancer Research and Development Center. PP2 and pNPP were purchased from Calbiochem and Sigma, respectively.

cDNA constructs

The plasmids encoding WT DEP-1, the C/S and D/A mutants were provided by Nicholas Tonks (Cold Spring Harbor Laboratory, NY, USA).(234) The WT and mutant Myr-DEP-1 cDNA constructs were generated as previously described.(160) All of the Y/F and E/Q DEP-1 mutants were made using the QuikChange XL site-directed mutagenesis kit (Stratagene). The mutagenic primers used are described

below. The Src Y527F and Src Y527F/R175L plasmids were provided by Jeroen den Hertog (Hubrecht Institute, Utrecht, Netherlands), whereas the SrcRF (K295R/Y527F) and WT Src cDNA constructs used in these studies were obtained from Drs. Joan Brugge (Harvard Medical School, USA) and Stéphane Laporte (McGill University Health Centre, Montreal, Canada), respectively (206). WT pRK5-Fyn is from Filippo Giancotti (Sloan-Kettering Institute for Cancer Research, New York, USA) and was obtained through Addgene. The GST-Src SH2 and GST-Src SH3 domain constructs (in pGEX-2TK) were generated using standard PCR procedures.

Generation of Y/ F mutants in Myr-DEP-1 D/A and full-length DEP-1

The mutagenic primers (Invitrogen) used to generate the various Myr-DEP-1 D/A Y/F mutants were the following:
5'-AACTGAAGACAGGCTTCTTGTCCATCGTCATGGATCC-3'(Y1045F), 5'-GTCAAGGGAAAGACTTCGTTGGAGCAATCCCTGTGGAT-3' (Y1094F), 5'-GAAGCTCCTGAAGATCTGTTTAAGGACTTCCTGACC-3' (Y1161F), 5'-CTGTGACTTTGGCTTGGCCCGGGATATTTTTAAAGATCCAG-3' (Y1268F), 5'-GATATTTATAAAGATCCAGATTTTGTCAGAAAAGGAGATGCTCGC-3' (Y1288F), 5'- TGCTCAGCAGGATGGCAAAGACTTCATTGTTCTTCCGA-3'(Y1311F), 5'-GACCCCAAATTCCATTTTGACAACACAGCAGGAATCAGTC-3' (Y1320F). The Y1311F/Y1320F mutant was made on the Y1311F mutant using the Y1320F mutagenic primer. Y1311F, Y1320F and Y1311F/Y1320F mutations were also generated on the full-length WT DEP-1 expressing vector. The E1321Q mutation was generated in DEP-1 WT using the following mutagenic primer: 5'-GCGATG ACAATCTATCAAAACCTTG-CGCCCGTG -3' (E1321Q). The presence of all mutations was confirmed by DNA sequencing.

Cell culture

HEK 293, HEK 293T, SYF mouse embryonic fibroblasts (MEFs) and bovine aortic endothelial cells (BAECs from Lonza; passages 1 to 8) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen) and 50 μg/ml gentamycin. Human umbilical vein endothelial cells (HUVECs; Cascade Biologics, Invitrogen) were cultured (passages 1 to 4) on gelatin (0.2%)-coated tissue culture dishes in M200 (Invitrogen) supplemented with 2% FBS, hydrocortisone, EGF, bFGF, heparin (LSGS kit; Invitrogen) and 50 μg/ml gentamycin.

DNA transfection conditions

Transfections were performed using either the standard calcium phosphate method or Lipofectamine 2000/Lipofectin according to the manufacturer's recommendations (447). In general, 5-10 μg of cDNA constructs were transfected in HEK 293 cells. In HEK 293T cells, 2 μg of DEP-1 constructs were co-transfected with 1 μg of SrcY527F (Figures 2E, 2F, Supplemental Figures 1A and 2B), while 3 or 4 μg were transfected to study the phosphorylation of endogenous Src and of transfected DEP-1 and VEGFR2 (Figure 3B, C and E). MEFs were transfected with DEP-1 D/A (10 μg) alone or with WT Src or Fyn constructs (5 μg). BAECs and HUVECs were transfected with 6-8 μg/10 cm-dish and 10 μg/6 cm-dish, respectively.

Immunoprecipitation and Western blotting

Cell lysates (200-500 μg) were incubated with antibodies for 1.5h at 4^{o}C, followed by incubation with protein A- or G-conjugated Sepharose beads (Amersham Biosciences/GE Healthcare) for an additional 1.5h. Beads were washed 3 times with lysis buffer and then denatured in 1x Laemmli sample buffer. In vitro Src kinase assay was performed using the Millipore kit as described.(160) Proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred onto Hybond-C extra membranes (Amersham Biosciences/GE Healthcare) and immunoblotted according to standard procedures as previously described (160).

GST pull-down assay

GST fusion proteins were purified as described (160). Five μg of GST, GST-Src SH2 or GST-Src SH3 fusion proteins were incubated for 30 min at 4 °C with Glutathione-conjugated Sepharose beads (Amersham Biosciences/ GE Healthcare). After three washes with lysis buffer, GST proteins coupled to beads were incubated for 1.5h at 4 °C with lysates (500 μg) of transfected HEK 293 cells. Protein complexes were washed 3 times with lysis buffer, resolved by SDS-PAGE and detected by Western blotting.

Cell adhesion assay

Twenty-four well plates were coated with Matrigel (200 μg/well) for 2h at 37°C, washed twice with PBS and then blocked with BSA (0.5mg/ml in PBS) for 1h at 37°C. Transfected HUVECs (5×10^4 cells) were seeded in duplicates in M200 medium and allowed to adhere in the presence of VEGF (10 ng/ml) for 30 min at 37°C. Cells were fixed with formalin for 20 min and then stained with crystal violet (0.1%) in 20% (v/v) methanol overnight. For quantification, cell counts were performed for each condition on 6 photographs/well taken at ×10 magnification using a Nikon Eclipse TE300 microscope and a Photometrics CoolSNAP camera.

DNA transfection, cell stimulation and lysis

HEK 293 and HEK 293T cells were seeded at 0.8 and 1.2×10^6 cells/10-cm dish, respectively, and transfected using the standard calcium phosphate method. Cells were lysed 48h post-transfection in a 50 mM HEPES [pH 7.5] buffer (160), as for all other experiments described below. MEFs (1.7×10^6 cells/10-cm dish) were transfected in OptiMEM (Invitrogen) using Lipofectamine 2000. BAECs (2×10^6 cells/10-cm dish) and HUVECs ($5.25 – 6.3 \times 10^5$ cells/6-cm dish) were transfected in OptiMEM with Lipofectamine 2000 and Lipofectin, respectively. See Supplemental

data section for amounts of DNA used. Medium was replaced the next morning with supplemented DMEM (BAECs) or M200 (HUVECs). Cells were serum-starved the next day for 16h (BAECs), or 42h later for 6h (HUVECs), and then stimulated with VEGF 50 ng/ml for the indicated times before cell lysis. Alternatively, HUVECs were collected for biological assays.

RNAi transfection

HUVECs (3×10^4 cells/cm^2 for cell signaling experiments and $2.2 - 2.5 \times 10^4$ cells/cm^2 for biological assays) were transfected with DEP-1 (Hs_PTPRJ_3_HP) and AllStars control siRNAs from Qiagen at a final concentration of 75 nM in M200 medium using Dharmafect reagent #4 (Dharmacon). Medium was replaced 16h post-transfection with supplemented M200. At 42h post-transfection, cells were serum-starved for 6 hours in M200 and stimulated with VEGF as described above. Alternatively, cells were submitted to biological assays.

In vitro phosphatase assay

Immunoprecipitated DEP-1 was resuspended in 50 µl of a 50 mM HEPES [pH 7.4] assay buffer containing BSA (0.1 mg/ml) and DTT (3mM) and transferred into a 96-well plate. Paranitrophenyl phosphate (pNPP; 50 µl of a 50 mM stock in water) was added to the wells and incubated 5-15 min at room temperature. Absorbance was read at 405 nm with a Victor^3 V fluorescence reader (PerkinElmer). pNPP hydrolysis after 5 min of reaction is reported in the graph of Figure 3D.

Capillary-like formation on Matrigel

Ice-cold Matrigel (BD Biosciences; 50 □l/well) was added to flat-bottomed 96-well plates and allowed to solidify 1h at 37^{o}C. HUVECs (2×10^4 cells/well) were seeded in duplicates on Matrigel and incubated 5-6h at 37°C in supplemented M200.

Capillaries were fixed in formalin buffer and photographs were taken (6/well) at ×10 magnification. The complexity of the network was quantified by measuring the length of tubes formed and the number of branches at connecting nodes.

Matrigel Invasion

Transwell filters (polycarbonate membrane, 8 μm-pore size; Corning Brand, Fisher) were coated with 50 μl/filter of Matrigel (2 mg/ml) that was allowed to solidify for 2h at 37°C. Transfected HUVECs (5×10^5 cells) were resuspended in 400 μl of M200 medium without LSGS and seeded in duplicate in the top chamber. The lower chamber was filled with M200 medium containing VEGF (50 ng/ml). Cells were incubated for 20h and then fixed with phosphate-buffered formalin for 20 min before staining with crystal violet (0.1% in 20% (v/v) methanol) for a minimum of 60 min. The Matrigel layer and cells remaining on top of the filter were wiped off. Only cells that went through the filter pores were visualized and counted.

Endothelial permeability assay

Six hours after transfection, HUVECs were seeded onto Transwell permeability inserts (6,5mm diameter, 1,0 μm pore size; BD Falcon, BD Biosciences) pre-coated with rat tail collagen type I (50μg/ml). After 46h, the cells were serum-starved for 1h in M200 medium and then stimulated with VEGF (50ng/ml) in the presence of FITC-Dextran (40 kDa size, 1 mg/ml). Permeability was determined by measuring the fluorescence at 520 nm (498 nm excitation) that was emitted from 50 μl-medium aliquots taken from the bottom chambers and diluted with 200 μl of M200 medium. Fluorescence was detected with a Victor^3 V fluorescence reader (Perkin-Elmer).

In vitro DEP-1/Src associatio

Purified GST-DEP-1_{IC} encompassing the intracellular domain (a.a. 997-1337) was cleaved with Thrombin (Calbiochem, Millipore) for 1h at R.T. Thrombin was removed from the eluate using p-aminobenzamidine-agaraose beads (Sigma) for 30 min at 4°C. Approximately 0.3 μg of DEP-1_{IC} was incubated or not with 10 ng of

purified recombinant Src (Millipore) and submitted to a cold kinase assay (according to Millipore's instructions) for 20 min at 30°C. Lysis buffer was added at the end of the reaction and proteins were incubated for 2h with DEP-1 goat antibodies, which recognize the intracellular domain, and then further incubated with Protein G Plus/Protein A- Agarose beads blocked with BSA (Calbiochem, Millipore) for an additional 2h. Co-immunoprecipitating Src was revealed following Western blotting with Src antibodies.

Immunofluorescence microscopy

HUVECs (2×10^5 cells) were plated onto gelatin-coated glass coverslips in 24-well inserts 6h after RNAi transfection. Cells were further incubated 24h, serum-starved in M200 for 2h and then stimulated with VEGF (50 ng/ml) for 30 min before fixation in formaldehyde (3,7% (v/v) in PBS) for 20 min and a 10 min-permeabilization in Triton X-100 (0. 2%). Cells were next blocked 30 min in FBS (8% solution (v/v) in PBS) and incubated 1h with the β-catenin antibody (1:50), followed by a 45 min incubation with the Alexa Fluor 488-coupled mouse secondary antibody (1:1000). Coverslips were mounted in ProLong Gold antifade reagent containing DAPI (Invitrogen/Molecular Probes).

Data analysis

Statistical significance was evaluated with the Mann-Whitney rank sum test using the SPSS software. P-values of less than 0.05 were considered to be significant.

Results

DEP-1 is tyrosine-phosphorylated in a Src family kinase-dependent manner.

Activation of Src by the protein tyrosine phosphatase PTPε has been linked to its tyrosine phosphorylation and its capacity to associate with the kinase (299, 304). In order to determine if a similar mechanism was involved in the ability of DEP-1 to activate Src, we first confirmed that DEP-1 was tyrosine phosphorylated and identified the kinases potentially involved. As shown in Figure 1, WT Myr-DEP-1 and a catalytically inactive and substrate trapping mutant (Myr-DEP-1 D/A), which encompass the intracellular portion of DEP-1 fused to the myristylated sequence of Src, were constitutively phosphorylated when expressed in HEK 293 cells (Figure 1A-B). However, the phosphorylation of WT Myr-DEP-1 was much weaker, indicating that it was subject to auto-dephosphorylation, similarly to what was observed for PTPε (299). Consistent with these observations, the phosphorylation of full-length WT DEP-1 was enhanced when cells were incubated with pervanadate, a general PTP inhibitor (Figure 1C). To determine if SFKs were implicated in this constitutive phosphorylation event, HEK 293 cells expressing Myr-DEP-1 D/A were treated with a pharmacological SFK inhibitor (PP2), DMSO as vehicle control, or co-transfected with a dominant negative mutant form of Src (RF-Src). As shown in Figure 1D, PP2 treatment or expression of RF-Src decreased the tyrosine phosphorylation of DEP-1, while co-transfection of a constitutively active Src mutant (Src Y527F) with either full-length WT DEP-1 or the DEP-1 C/S mutant increased their phosphorylation (Figure 1E). The C/S mutant is catalytically inactive but has virtually no ability to trap substrates compared to the DEP-1 D/A mutant,(160) and thus is not similarly protected from dephosphorylation by other endogenous phosphatases (448). Lastly, Figure 1F shows that phosphorylation of full-length DEP-1 D/A is barely detectable in Src/Yes/Fyn-null fibroblasts (SYF MEFs), but rescued

by re-expression of WT Src or Fyn. These results thus demonstrate that DEP-1 can be tyrosine-phosphorylated in a SFK-dependent manner.

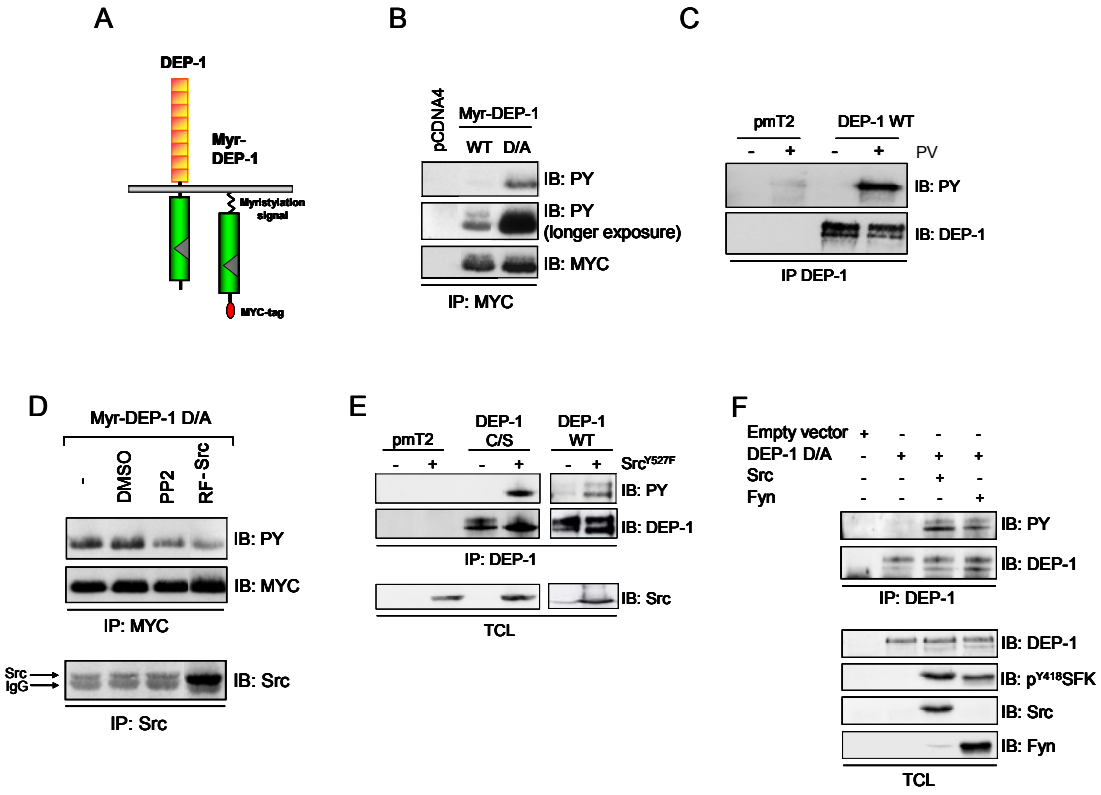

Figure 1: DEP-1 is tyrosine phosphorylated in a SFK-dependent manner.

(A) Structure of full-length DEP-1 and Myc-tagged Myr-DEP-1. (B) Myr-DEP-1 WT and inactive Myr-DEP-1 D/A mutant were expressed in HEK 293 cells. Tyrosine phosphorylation of immunoprecipitated DEP-1 was analyzed by immunoblotting (*IB*) with the PY99 phosphotyrosine antibody (*PY*). The membrane was stripped and reprobed with the Myc antibody to detect equivalent levels of immunoprecipitated Myr-DEP-1. (C) Tyrosine phosphorylation of full-length WT DEP-1 is increased by pervanadate (*PV*) treatment of HEK 293T cells transfected with empty vector (pmT2) or full-length WT DEP-1. At 48h post-transfection, cells were incubated or not with PV (100 μM) for 20 min before cell lysis. DEP-1 was immunoprecipitated (*IP*) with the clone 143-41 mouse antibody, and its tyrosine phosphorylation determined by immunoblotting with the PY99 antibody. The membrane was stripped and reprobed with the DEP-1 antibody (clone 143-41) to show similar levels of immunoprecipitated DEP-1. (D) Tyrosine phosphorylation of Myr-DEP-1 D/A is impaired by PP2 or by a Src dominant negative mutant. HEK 293 cells were transfected with Myr-DEP-1 D/A and incubated with either PP2 (5 μM; 30 min) or vehicle (DMSO), or co-transfected with a Src dominant negative mutant (RF-Src). Tyrosine phosphorylation of immunoprecipitated DEP-1 was detected as described in *B*. Src-RF expression was determined following immunoprecipitation with the mouse Src antibody (GD11 clone) and immunodetection with the rabbit clone 36D10. (E) Tyrosine phosphorylation of DEP-1 is increased by co-expression of a constitutively active Src mutant. HEK 293T cells were transfected with empty vector (pmT2), WT DEP-1, or the C/S mutant in the presence or not of active Src Y527F. Tyrosine phosphorylation of immunoprecipitated DEP-1 was determined as described above. Immunoblotting of total cell lysates with the Src antibody (clone GD11) reveals equivalent Src Y527F expression levels. (F) WT Src and Fyn promote DEP-1 D/A tyrosine phosphorylation. SYF MEFs were transfected with empty vector (pmT2), the DEP-1 D/A mutant alone, or in combination with either WT Src or Fyn encoding vectors. Phosphorylation of immunoprecipitated DEP-1 is revealed as described above. Immunoblotting of total cell lysates with the indicated antibodies demonstrate similar amounts of DEP-1 and SFKs in the various conditions. All results are representative of 3 independent experiments.

DEP-1 C-terminal Y1311 and Y1320 are phosphorylation sites and associate with Src.

To identify DEP-1 phosphorylated site(s), residues corresponding to SFK consensus phosphorylation sites and/or potential SH2-domain binding sites were mutated in Myr-DEP-1 D/A (Figure 2A). The resulting Y/F mutants were expressed in HEK 293

cells and their phosphorylation level relative to the "non-mutated" Myr-DEP-1 D/A was evaluated by immunoblotting total cell lysates with the anti-phosphotyrosine antibody PY99. Figure 2B reveals that the Myr-DEP-1 D/A Y1311F and Y1320F mutants were the least phosphorylated. Consistently, mutation of both residues led to the complete abrogation of Myr-DEP-1 D/A tyrosine phosphorylation (Fig. 2C). These two tyrosine residues were the only tyrosine-phosphorylated sites detected by mass spectrometry analysis performed on Myr-DEP-1 D/A (data not shown).

Analysis of the amino acid sequences downstream of Y1311 and Y1320 suggested that these sites might be Src SH2-domain binding sites. To test this, we first performed in vitro association experiments and investigated the ability of GST alone, GST-Src-SH2 or GST-Src-SH3 fusion proteins coupled to Glutathione-Sepharose beads to associate with Myr-DEP-1 WT, Myr-DEP-1 D/A or the Myr-DEP-1 D/A Y/F mutants from transfected HEK 293 cell lysates. The highly tyrosine-phosphorylated Myr-DEP-1 D/A associated with the GST-Src-SH2 fusion proteins with a strong affinity, while mutation of Y1311 and Y1320 residues abolished this interaction (Fig. 2D). We further showed that recombinant Src directly associated with the purified phosphorylated intracellular domain of DEP-1 in vitro (Figure 2E), supporting the conclusion that DEP-1 phosphorylated on Y1311 and Y1320 directly associates with the Src SH2 domain.

To demonstrate that the phosphorylated DEP-1 Y1311 and Y1320 are also implicated in the binding of Src in vivo, DEP-1 C/S and the C/S Y1311F, Y1320F, and Y1311F/Y1320F mutants were co-expressed in HEK 293T cells with constitutively active Src (Src Y527F) to enhance the phosphorylation of DEP-1, as previously done (Fig. 1E). Figure 2F shows that full-length DEP-1 C/S co-immunoprecipitated with Src. Mutations of Y1311 or Y1320 resulted in the decreased association of DEP-1 to Src, with the mutation at Y1320 having a slightly stronger effect. In the converse experiment, DEP-1 C/S failed to associate with a Src SH2 domain mutant (Y527F/R175L), further confirming that DEP-1 associates with Src through its SH2 domain (Fig. 2G). Altogether, these results demonstrate that the

tyrosine phosphorylation of DEP-1 on Y1311 and Y1320 can mediate the association of Src via its SH2 domain, and that Y1320 may be a slightly preferred binding site.

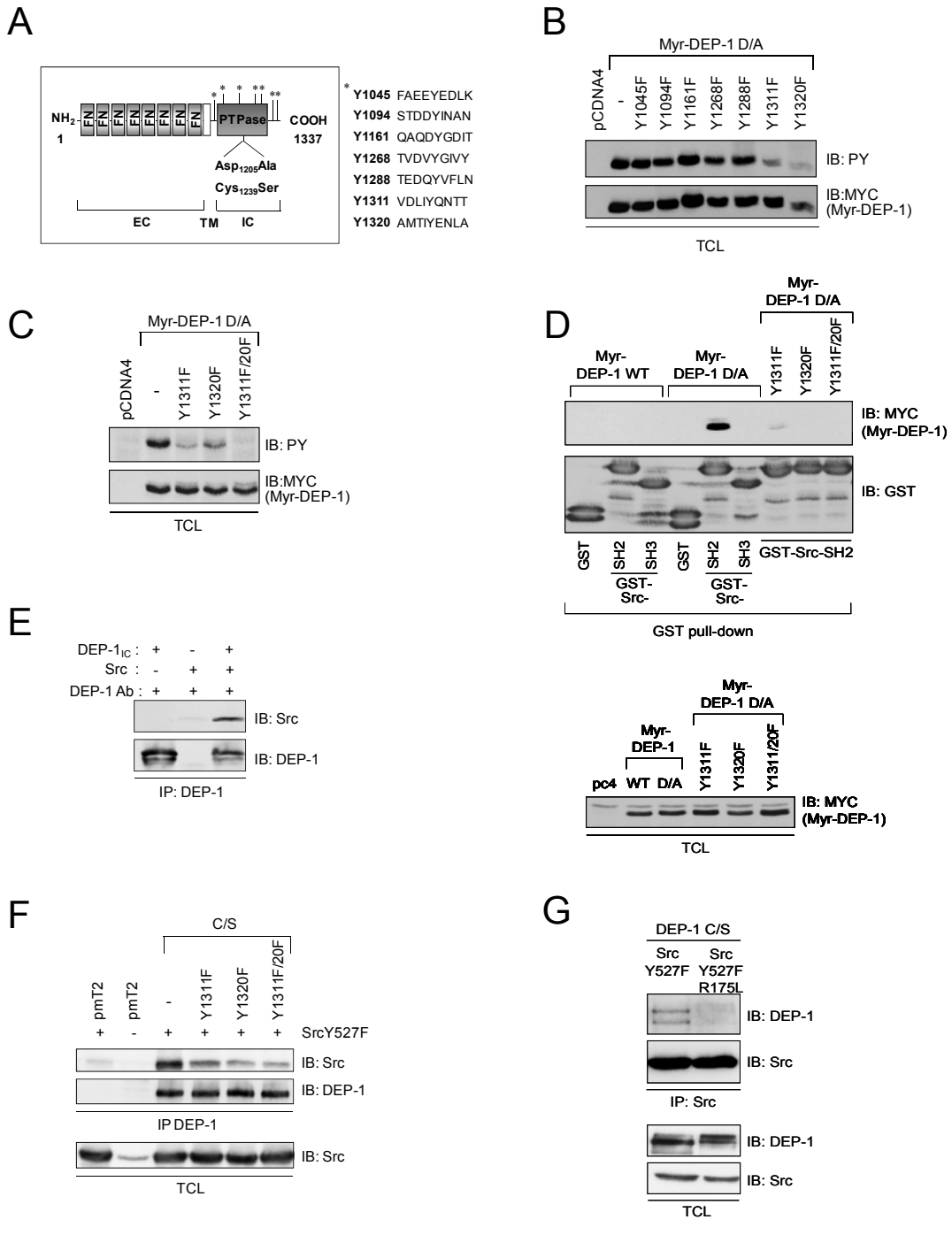

Figure 2: **DEP-1 Y1311 and Y1320 are major phosphorylation sites that associate with Src via its SH2 domain.**

(A) Diagram representing the potential SFKs tyrosine phosphorylation sites in the intracellular domain of DEP-1 according to published consensus sequences or Scansite predictions (http://scansite.mit.edu/). FN, Fibronectin type III-like repeats; EC, extracellular; TM, transmembrane; IC, intracellular (B) HEK 293 cells were transfected with "non-mutated" (-) Myr-DEP-1 D/A or the various Y/F mutants. Their tyrosine phosphorylation was detected by immunoblotting (IB) total cell lysates (TCL) with the PY99 antibody (PY). Expression levels of transfected Myr-DEP-1 were detected with a Myc antibody. (C) HEK 293 cells were transfected with Myr-DEP-1 mutated at both Y1311 and Y1320, which is not detectably tyrosine phosphorylated. (D) DEP-1 associates with the Src SH2 domain via its phosphorylated Y1311 and Y1320 residues. Lysates of HEK 293 cells transfected with the indicated constructs were incubated with GST-Src-SH2, GST-Src-SH3 or with GST alone. Association of DEP-1 with Src domains was detected by immunoblotting with the MYC antibody. The equivalent amount of GST fusion proteins used in the pull-down-assay is detected with a GST antibody. Lower panel, Lysates of transfected HEK 293 cells were immunoblotted with the MYC antibody to show the similar expression level of DEP-1 constructs. (E) In vitro association of recombinant Src with the purified intracellular domain of DEP-1 (DEP-1_{IC}) previously phosphorylated by Src in vitro. DEP-1_{IC} was immunoprecipitated with DEP-1 goat antibodies and associated Src was immunodetected using the 36D10 clone. (F) The association of DEP-1 C/S and DEP-1 C/S Y/F mutants to Src was investigated in HEK 293T cells co-transfected with constitutively active Src, to induce maximal tyrosine phosphorylation of DEP-1. The co-precipitation of Src with DEP-1 and the equal expression of Src Y527F were detected by immunoblotting with a Src antibody (GD11 clone). (G) Mutation in the Src SH2 domain abrogates its ability to associate with DEP-1. HEK 293T cells were co-transfected with DEP-1 C/S and activated Src Y527F or the Src Y527F/R175L SH2 domain mutant. Src was immunoprecipitated with the mouse GD11 mouse clone, and associated DEP-1 was detected with the goat antibody. Immunoblotting of total cell lysates with the DEP-1 goat antibody or the Src GD11 antibody reveal that equal amounts of Src and DEP-1 constructs were expressed. All results are representative of at least 3 independent experiments.

Mutation of Y1311 and Y1320 impairs DEP-1-mediated activation of Src in HEK 293T cells, without affecting PTP activity.

To determine if the binding of the Src SH2 domain to phosphorylated DEP-1 Y1311 and Y1320 could be involved in a phospho-displacement mechanism leading to Src

activation, we first investigated the ability of WT DEP-1 and the Y/F mutants to activate Src in HEK 293T cells. Figure 3A shows that transfection of increasing amounts of WT DEP-1 led to the progressive dephosphorylation of inhibitory Y529 while increasing the phosphorylation of Y418 and kinase activity. Interestingly, as levels of expression reached a maximum, Y418 phosphorylation and kinase activity also started to decrease, suggesting that DEP-1 can also dephosphorylate Src on Y418 in these conditions, as previously observed in vitro (318). This experiment thus indicated that the level of DEP-1 expression is crucial in determining its substrate specificity and function. We next investigated the consequences of expressing DEP-1 Y/F mutants on Src phosphorylation using optimal transfection conditions resulting in the activation of Src by WT DEP-1, as determined in Figure 3A. Results show that Src Y418 phosphorylation was slightly decreased upon expression of the DEP-1 Y1311F mutant when compared to cells expressing WT DEP-1, while changes in the phosphorylation status of Src Y529 were barely visible. In contrast, expression of the DEP-1 Y1320F or Y1311F/Y1320F mutants strongly impaired phosphorylation of Y418 and dephosphorylation of Y529, in a manner comparable to that observed following expression of the catalytically inactive DEP-1 C/S mutant (Figure 3B). Using a validated phosphospecific DEP-1 antibody recognizing phosphorylated Y1320 (see Supplemental Figure 1), the constitutive phosphorylation of WT DEP-1 and of the Y1311F mutant was detected in HEK 293T cells and correlated with their ability to upregulate Src Y418 phosphorylation (Figure 3C). The DEP-1 C/S catalytically inactive mutant was also constitutively phosphorylated, demonstrating that tyrosine phosphorylation is not sufficient for Src activation and that the catalytic activity of DEP-1 is also required. These results thus suggest that phosphorylated DEP-1 Y1311 and Y1320 associate with Src, and that this is required for Src activation. However, as phosphorylation of PTPs can modulate their catalytic activity,(449) the consequences of mutating Y1311 and Y1320 on the catalytic activity of DEP-1 were also investigated. For this, WT DEP-1 and the various Y/F mutants were co-transfected with activated Src (Y527F) in HEK 293T cells to

promote tyrosine phosphorylation of WT DEP-1 (as shown in Figure 1E). An in vitro PTP assay using pNPP as a substrate was performed on immunoprecipitated DEP-1. The results reveal that the Y1311F, Y1320F or double Y1311F/Y1320F mutations did not significantly interfere with the catalytic activity of DEP-1 in these experimental conditions (Figure 3D). To further confirm this result, dephosphorylation of VEGFR2, previously identified as a DEP-1 substrate, (149, 160) was monitored in VEGF-stimulated HEK 293T cells co-expressing VEGFR2 with either WT DEP-1, DEP-1 Y1311F/Y1320F or the C/S mutant in conditions similar to those of Figure 3B. Immunoblotting with a general phosphotyrosine antibody showed that the DEP-1 Y1311F/Y1320F mutant was as efficient as WT DEP-1 at reducing the phosphorylation of VEGFR2, while the C/S mutant had no effect, as expected (Figure 3E). On the basis of these results, we conclude that the phosphorylation of DEP-1 on Y1311 and Y1320 is not involved in the regulation of its catalytic activity. This then strongly suggests that the decreased dephosphorylation/activation of Src in cells expressing the DEP-1 Y1311F/Y1320F mutant is not due to reduced catalytic activity of the mutant but rather to its lost ability to associate with Src.

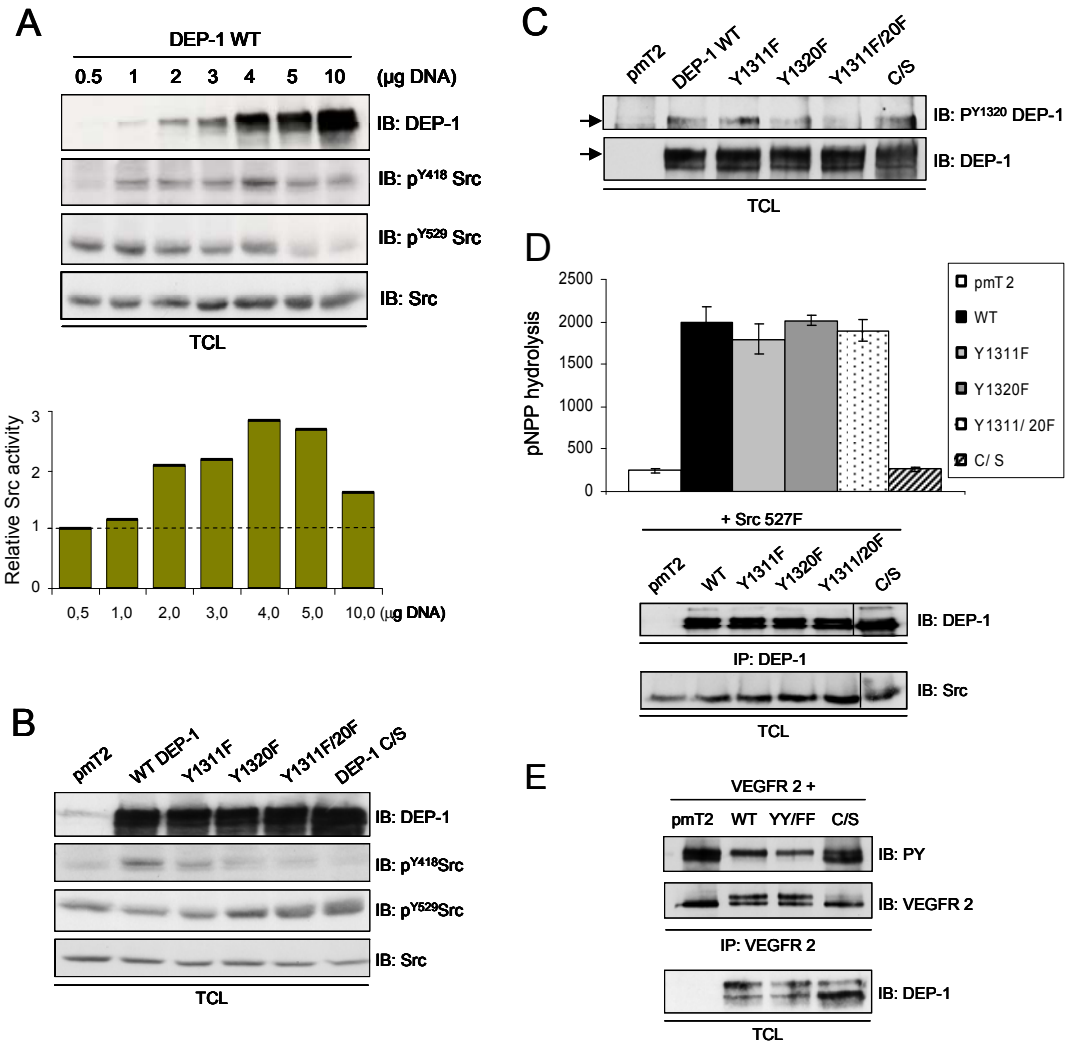

Figure 3: Mutation of Y1311 and Y1320 impairs DEP-1-mediated activation of Src in HEK 293T cells, without affecting PTP activity.

(A) HEK 293T cells were transfected with increasing amounts of WT DEP-1 cDNA vector. The phosphorylation level of Src on Y529 and Y418 was determined by immunoblotting total cell lysates (*TCL*) with the corresponding phosphospecific antibodies. *Lower panel,* Src was immunoprecipitated from the corresponding cell lysates shown in A with the Src GD11 antibody and its kinase activity determined in vitro using a Src peptide substrate with [γ-^{32}P]ATP as previously done (160). Based on these results, all other transfection experiments were performed with 3-4 μg of DEP-1 plasmids to achieve optimal Src activation in these cells. This result is representative of 4 independent experiments. (B) HEK 293T cells were transfected with empty vector (pmT2), WT DEP-1 and the indicated mutants, and the phosphorylation level of Src on Y529 and Y418 analyzed as described above. (C) DEP-1 is constitutively tyrosine phosphorylated in HEK 293T cells. Total cell lysates of HEK 293T cells transfected with equal levels of WT DEP-1 and the various mutants were immunoblotted with a phosphospecific antibody detecting p^{Y1320} DEP-1. (D) The catalytic activity of DEP-1 is not affected by the various mutations reducing its tyrosine phosphorylation. WT DEP-1 and mutants were co-expressed with activated Src Y527F in HEK 293T cells to enhance DEP-1 phosphorylation. DEP-1 was immunoprecipitated from 200μg of HEK 293T cell lysates (not containing phosphatase inhibitors) and submitted to the pNPP assay as described in the Material and Methods section. Empty vector (pmT2) and DEP-1 catalytically inactive C/S mutant represent negative controls. pNPP hydrolysis after 5 min of reaction is reported in this graph. This result represents the average of 3 independent experiments. *Lower panel,* Western blot analysis reveals similar levels of expressed WT DEP-1, mutants, and constitutively active Src in the various conditions tested. *$P<0.05$ (E) Mutant DEP-1 is as effective as WT DEP-1 at dephosphorylating VEGFR2. HEK 293T cells co-transfected with VEGFR2 and either empty vector (pmT2), WT DEP-1, DEP-1 Y1311F/Y1320F (YY/FF) or the C/S mutant were stimulated with VEGF (50 ng/ml) for 5 min. Phosphorylation of immunoprecipitated VEGFR2 was detected using a general phosphotyrosine antibody (PY99). Similar DEP-1 expression levels are shown. This result is representative of 2 independent experiments.

DEP-1 Y1311 and Y1320 are required for VEGF-dependent Src activation in endothelial cells.

We previously demonstrated that DEP-1 is required for VEGF-induced Src activation in primary cultures of endothelial cells (160). Overexpression of DEP-1 reveals here that similarly to what was observed in HEK 293T cells (Figure 3A), moderate versus high expression levels of DEP-1 in endothelial cells also led to opposite effects on

Src activation and phosphorylation of its substrate VE-cadherin (Supplemental Figure 2A) (135, 450). This is consistent with the notion that higher expression of endogenous DEP-1 is observed in post-confluent or quiescent endothelial cells in vitro and in vivo, while its expression is decreased to moderate levels in growing and migrating cells (149, 262). To find out if Src activation also relied on DEP-1 Y1311 and Y1320 in VEGF-stimulated endothelial cells, we therefore investigated the consequences of expressing moderate amounts of WT and DEP-1 Y/F mutants in primary cultures of bovine aortic endothelial cells (BAECs). Figure 4A shows that VEGF stimulation of control cells (pmT2-transfected) led to the dephosphorylation of Src Y529, while this was observed constitutively in cells overexpressing WT DEP-1. In contrast, a progressive decrease in Y529 dephosphorylation induced by VEGF was observed when Y1311F, Y1320F and Y1311F/Y1320F mutants were expressed, the latter being the most efficient at inhibiting Src Y529 dephosphorylation. In these same conditions, the VEGF-induced phosphorylation of ERK1/2 was similarly decreased in cells overexpressing WT DEP-1 or the mutants, demonstrating that impaired dephosphorylation of Src in cells expressing the DEP-1 mutants was not due to differential VEGFR2-dependent signaling or to reduced phosphatase activity. Our results also demonstrated that the Y1311F and Y1320F mutants but even more strikingly the double Y1311F/Y1320F mutant abolished the Src-dependent phosphorylation of VE-cadherin in response to VEGF stimulation, confirming the role of DEP-1 Y1311/Y1320 in VEGF-dependent Src activation (Figure 4B). To strengthen this observation, the sequence downstream of Y1320 (Y^{1320}ENL), corresponding to a potent Src SH2 binding site, was mutated to Y^{1320}QNL (Supplemental Figure 3). Results show that the association of Src with the DEP-1 C/S E1321Q mutant was much reduced and correlated with the impaired ability of VEGF to induce the dephosphorylation of Src Y529 and the phosphorylation of VE-cadherin in endothelial cells expressing this mutant. These experiments thus reveal the requirement of DEP-1 Y1311 and Y1320 for Src association and activation in VEGF-stimulated endothelial cells.

We next investigated if endogenous DEP-1 was phosphorylated in response to VEGF using DEP-1 phosphospecific antibodies. Figure 4C shows that phosphorylation of DEP-1 on Y1311 and Y1320 was rapid and transient, with maximal increases occurring within 2 minutes. A concurrent increase in Src Y418 phosphorylation was also observed over this time period. Importantly, similar kinetics of DEP-1 phosphorylation on Y1320 were observed in VEGF-stimulated BAECs overexpressing WT DEP-1, but phosphorylation was virtually absent in VEGF-stimulated cells overexpressing the Y1311F/Y1320F mutant where Src activation was found to be abrogated (Figure 4D). These results are thus consistent with our model-hypothesis that tyrosine phosphorylation of DEP-1 is part of the mechanism leading to Src activation, and that a phospho-displacement mechanism may be involved.

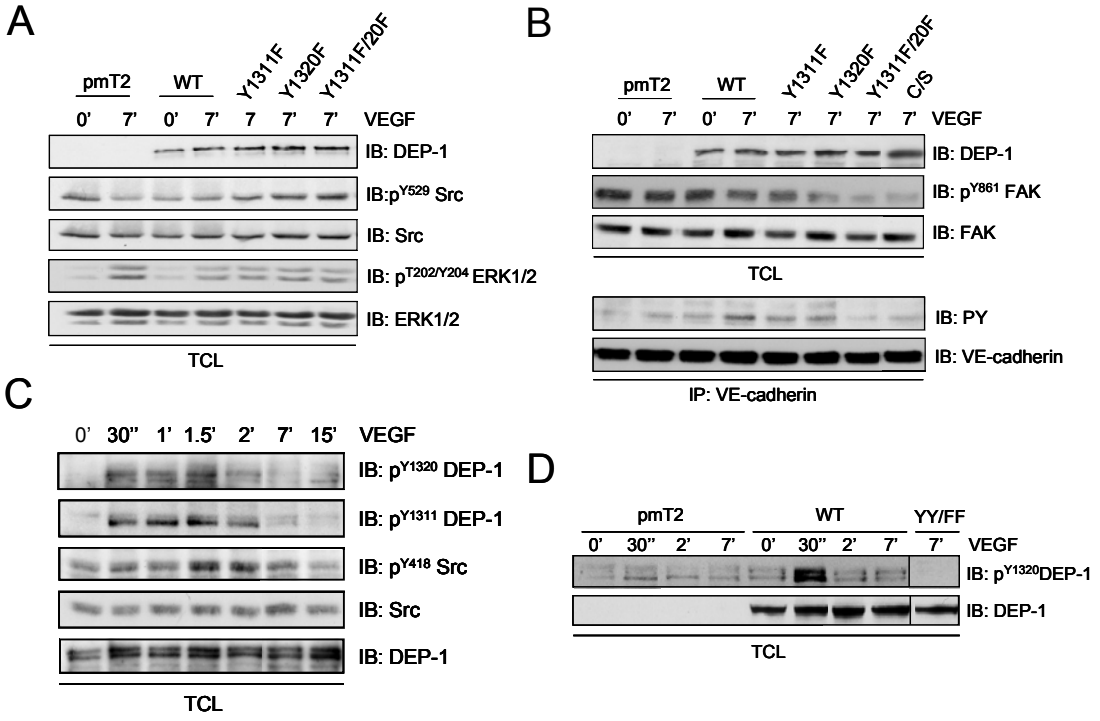

Figure 4: VEGF-dependent phosphorylation of DEP-1 on Y1311 and Y1320 mediate Src activation in endothelial cells.

(A) Bovine aortic endothelial cells (BAECS) were transfected with empty vector (pmT2), WT DEP-1 and the indicated mutants, serum-starved, and then stimulated with VEGF (50 ng/ml) for 7 min. Src dephosphorylation on Y529 was detected using a phosphospecific antibody recognizing phosphorylated Y529. VEGF-dependent signaling was monitored by detecting the activation of ERK1/2 using an antibody recognizing phosphorylated T202/Y204. Equivalent signaling is observed in cells expressing WT DEP-1 and mutants. (B) BAECs were treated as in *C*. The phosphorylation of VE-cadherin and FAK Y861 was investigated following the immunoprecipitation of VE-cadherin and its immunodetection with the PY99 antibody, and by immunoblotting total cell lysates with a FAK phosphospecific antibody recognizing Y861. These results are representative of 3 independent experiments. (C) HUVECs were serum-starved and stimulated with VEGF for the indicated times. Phosphorylation of endogenous DEP-1 is detected in total cell lysates (*TCL*) using phosphospecific antibodies detecting pY1311 and pY1320. The phosphorylation/activation status of Src on Y418 is determined by immunoblotting total cell lysates with the p^{Y418}Src antibody. Results suggest that VEGF-induced DEP-1 tyrosine phosphorylation is concomitant with Src activation. (D) The VEGF-mediated tyrosine phosphorylation of DEP-1 on Y1320 is enhanced in BAECs overexpressing WT DEP-1, but abrogated in cells expressing the Y1311F/Y1320F mutant (*YY/FF*).

DEP-1 promotes the VEGF-dependent remodeling of intercellular junctions and endothelial permeability via Y1311 and Y1320.

VEGF-dependent remodelling and loosening of intercellular adhesions are required to facilitate tip cell migration and invasion during sprouting angiogenesis, (18) but also represent key steps in the promotion of vascular permeability (443). Src is a major regulator of these processes, in part through the direct tyrosine phosphorylation of adhesion proteins including VE-cadherin, but also via the induction of a Src-VAV2-Rac-PAK pathway, resulting in the serine phosphorylation of VE-cadherin and its cellular internalization (114, 135, 137, 450, 451). To test if DEP-1 was implicated in the remodelling of cell-cell junctions in response to VEGF stimulation, the phosphorylation of VE-cadherin was first investigated in DEP-1-depleted HUVECs compared to control cells using antibodies recognizing VE-cadherin phosphorylated on Y658 and S665 (137, 450, 451). Consistent with decreased Src Y418

phosphorylation, Figure 5A shows that the VEGF-induced tyrosine phosphorylation of VE-cadherin, as detected with the p^{Y658}VE-cadherin antibody, was reduced in DEP-1-depleted cells. In addition, the Src-dependent phosphorylation/activation of the Rac GDP exchange factor VAV2 was similarly decreased and correlated with the inhibited phosphorylation of VE-cadherin on Serine 665 (Antibody specificity in Supplemental Figure 4). As phosphorylation of VE-cadherin correlates with its increased internalization and the weakening of its intracellular association with Catenins at cell-cell junctions, the above results suggested that the remodelling of cell-cell junctions and endothelial permeability might be defective in cells lacking DEP-1 expression (137, 450). Accordingly, immunostaining of HUVECs with a β-Catenin antibody revealed a "hairy" phenotype and the appearance of intercellular gaps representative of loosened cell-cell contacts upon stimulation of control cells with VEGF, while DEP-1-silenced cells maintained stable cell-cell junctions (Figure 5B). In agreement with this, HUVECs transfected with control siRNAs cells showed a significant increase in permeability in response to VEGF, while this was completely blocked in DEP-1-depleted cell monolayers (Figure 5C). Importantly, overexpression of WT DEP-1 in HUVECs promoted VEGF-dependent endothelial cell permeability compared to cells transfected with empty vector (pmT2) (Figure 5D). However, overexpression of the Y1311F/Y1320F mutant decreased permeability below the level detected in control cells, thus behaving as a dominant negative mutant. These results are consistent with a role for DEP-1 and its phosphorylated C-terminal tail in Src activation and the consequent phosphorylation of VE-cadherin in VEGF-stimulated endothelial cells, and highlight the role of DEP-1 in the control of endothelial permeability.

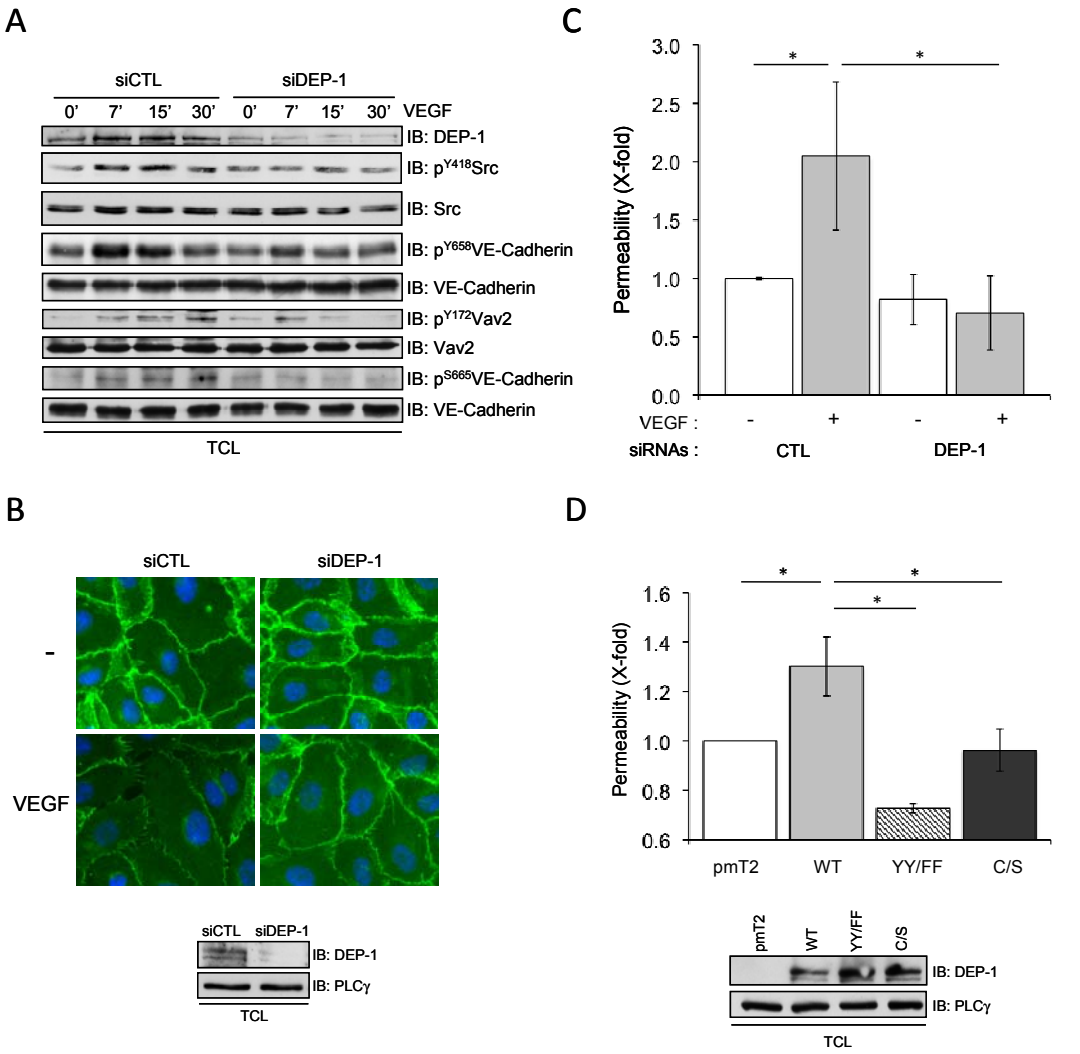

Figure 5: DEP-1 and its C-terminal Y1311 and Y1320 are essential mediators of VEGF-dependent endothelial permeability.

(A) DEP-1 promotes VEGF-dependent VE-cadherin phosphorylation. HUVECs were transfected with control (CTL) or DEP-1 siRNAs. Forty-two hours later, cells were serum-starved for 6h and stimulated with VEGF for the indicated times. The phosphorylation level of Src, VE-cadherin, and Vav2 was determined by immunoblotting with the indicated phosphospecific antibodies. Results are representative of at least 3 independent experiments. (B) DEP-1 is required for VEGF-induced loosening of cell-cell junctions. Control (CTL) and DEP-1-depleted cells were stimulated with VEGF (50 ng/ml) for 30 min and stained with β-Catenin and anti-mouse Alexa 488-secondary antibodies. *Lower panel,* RNAi-mediated decrease in DEP-1 expression levels. (C) DEP-1 mediates VEGF-induced endothelial permeability. Control (CTL) and DEP-1-silenced cells were plated on collagen-coated inserts until the formation of a tight monolayer. Forty-six hours later, cells were serum-starved for 2h and then stimulated or not with VEGF (50ng/ml) for 30 minutes in the presence of FITC-dextran (in the upper chamber). VEGF-induced permeability was measured by detecting the fluorescence emitted by FITC-dextran present in aliquots that were collected from the bottom chambers. Data is presented as fold changes over unstimulated control (CTL) cells. Essays were conducted in triplicates and results are representative of 4 independent experiments. *$p<0.05$ (D) HUVECs transfected with pmT2 empty vector, WT DEP-1, the Y1311F/1320F (*YY/FF*) or C/S mutants were plated on collagen-coated inserts, serum-starved for 1h and then stimulated for 30 min with VEGF in the presence of FITC-dextran, as described above. Data is presented as fold changes over stimulated control (pmT2) cells. Assays were conducted in triplicates and results are representative of 3 independent experiments.

Efficient endothelial cell invasion and capillary formation require DEP-1 and its phosphorylated C-terminal tail

Src is also a key promoter of endothelial cell invasion and neovascularization.(114, 129) One potential candidate substrate mediating these effects is Cortactin, which allows branched actin polymerization and the stabilization of membrane protrusions during cell migration and invasion.(452) It is enriched in membrane protrusions of endothelial cells in a Src-dependent manner and is essential for VEGF-induced migration and in vivo angiogenesis.(453-455) Importantly, Cortactin tyrosine phosphorylation also correlates with its ability to promote cell migration and invasion.(452, 455, 456) Consistent with the role of DEP-1 via Y1311/Y1320 in the

activation of Src, the depletion of DEP-1 in HUVECs or the expression of the DEP-1 Y1311F/Y1320F mutant in BAECs resulted in the decreased tyrosine phosphorylation of Cortactin, as detected using a p^{Y421}Cortactin antibody (Figures 6A and 6B). In contrast, Cortactin phosphorylation was stimulated by overexpression of WT DEP-1 (Figure 6B). As previously reported,(454) Figure 6C shows that Cortactin was enriched in the membrane protrusions of control endothelial cells stimulated with VEGF, but this localization was impaired in the DEP-1-depleted HUVEC population. In agreement with these observations, the VEGF-dependent invasion of DEP-1-silenced HUVECs into Matrigel was strongly impaired (Figure 6D). Conversely, overexpression of WT DEP-1 stimulated this response over control cells (pmT2-transfected), while expression of the Y1311F/Y1320F or C/S mutants were unable to do so (Figure 6E). These results thus show that DEP-1 via Y1311/Y1320 is required for the proper tyrosine-phosphorylation and localization of Cortactin to membrane protrusions, and is an essential mediator of endothelial cell invasion triggered by VEGF. It is interesting to note here that VEGF-stimulated endothelial cells expressing high levels of DEP-1, which rather mimic the status of quiescent/post-confluent endothelial cells,(262, 457) were unable to invade compared to pmT2-transfected cells or cells expressing moderate amounts of DEP-1(Supplemental Figure 2B). These results are thus consistent with the opposite capacity of moderate versus high levels of DEP-1 to activate Src.

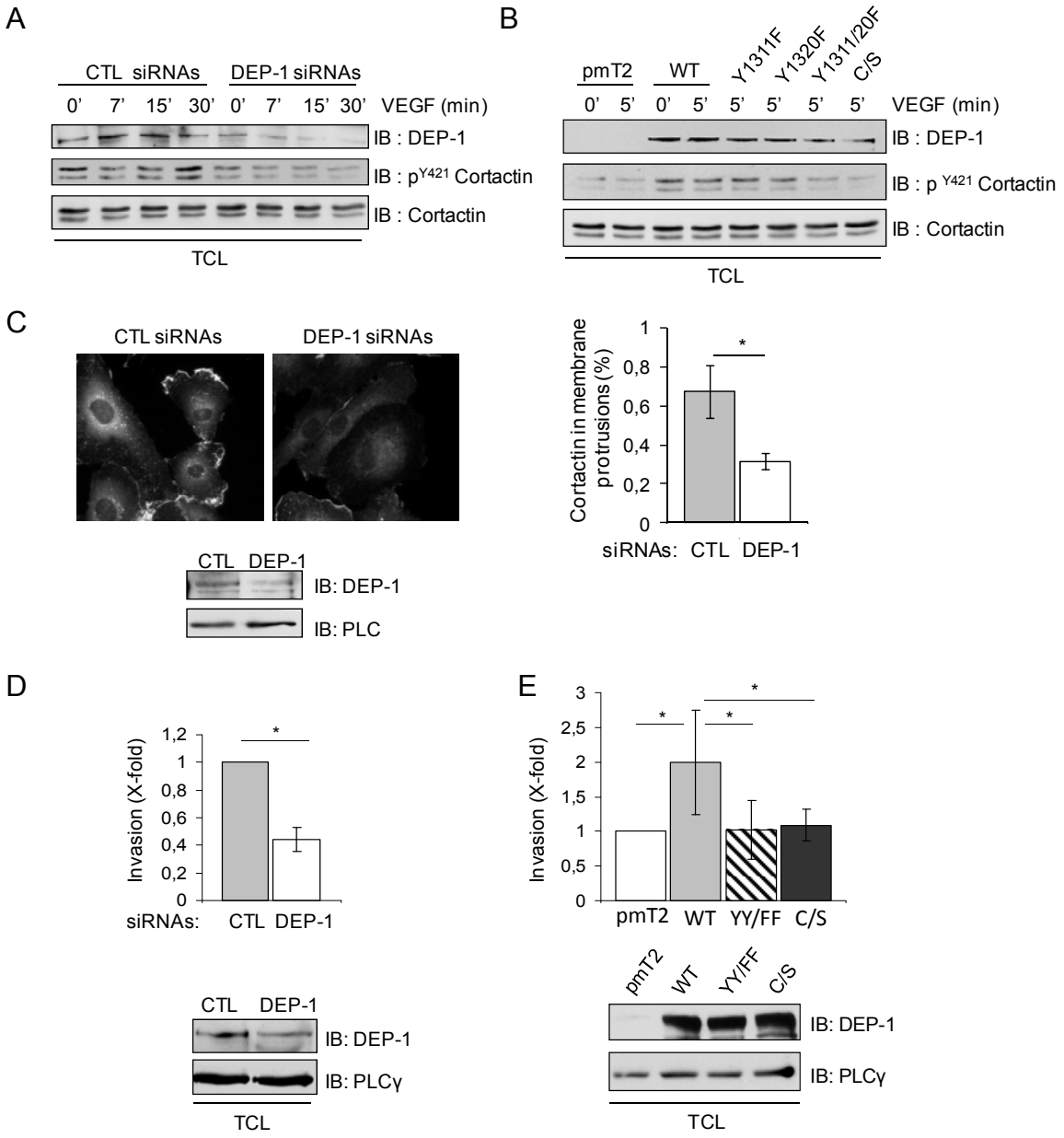

Figure 6: DEP-1 promotes VEGF-induced Cortactin tyrosine phosphorylation and endothelial cell invasion through Y1311 and Y1320.

(A) HUVECs were transfected with control (CTL) and DEP-1 siRNAs and then stimulated with VEGF (50 ng/ml) as indicated in legend to Figure 5A. Cortactin tyrosine phosphorylation was monitored by immunoblotting total cell lysates (TCL) with the p^{Y421}Cortactin antibody. DEP-1 expression and Cortactin protein levels were detected with the DEP-1 goat and Cortactin (4F11clone) antibodies, respectively. (B) BAECs were transfected with WT and mutant DEP-1 constructs, serum-starved and then stimulated 5 min with VEGF (50 ng/ml). Cortactin tyrosine phosphorylation and protein levels were monitored as described above. (C) DEP-1 is required for the localization of Cortactin in membrane protrusions. HUVECs transfected with control (CTL) or DEP-1siRNAs were plated on gelatin-coated glass coverslips, serum-starved, and then stimulated with VEGF (10 ng/ml) for 5 min before cell fixation. Immunostaining with Cortactin (4F11 clone) and Alexa Fluor 594-coupled mouse secondary antibodies is shown. The percentage of cells with enriched localization of Cortactin in membrane protrusions was evaluated in control and DEP-1-depleted cells in 4 microscopic fields at the 40 × magnification. *$p<0.05$ (D) HUVECs were transfected with control (CTL) or DEP-1 siRNAs and seeded in duplicates 48 h post-transfection on Transwell filter inserts previously coated with 50 µl of Matrigel (2 mg/ml). Cells were allowed to invade for 24h, and then processed to visualize and count cells on the lower side of the filter. Results ± SD have been normalized using the average from the control condition. *$p<0.05$ (E) HUVECs transfected with empty vector (pmT2), WT DEP-1, DEP-1 Y1311F/Y1320F (YY/FF) or DEP-1 C/S were plated in duplicates on Matrigel-coated filter inserts and processed as described above. Results are representative of 5 independent experiments. *$p<0.05$ All other results shown in this figure are representative of 3 independent experiments.

As could be expected based on the inhibition of Src and Cortactin phosphorylation, the plating of DEP-1-silenced HUVECs on Matrigel resulted in their impaired ability to reorganize and elongate branched capillary-like structures (Figure 7A). Expression of the DEP-1 Y1311F/Y1320F or C/S mutants also reduced the length of capillaries forming and significantly impaired branching compared to cells overexpressing WT DEP-1 (Figure 7B). Of note, there was no difference in the capacity of DEP-1-silenced cells or cells expressing the DEP-1 Y1311F/Y1320F mutant to adhere to Matrigel with that of control cells, demonstrating that the impaired invasion and capillary formation observed were not a consequence of decreased adhesion (Supplemental Figure 5). Altogether, the results presented herein demonstrate for the first time the central implication of endogenously expressed DEP-1 in the mediation

of Src-dependent angiogenic responses and reveal the essential contribution of DEP-1 Y1311 and Y1320 in the proper regulation of these events.

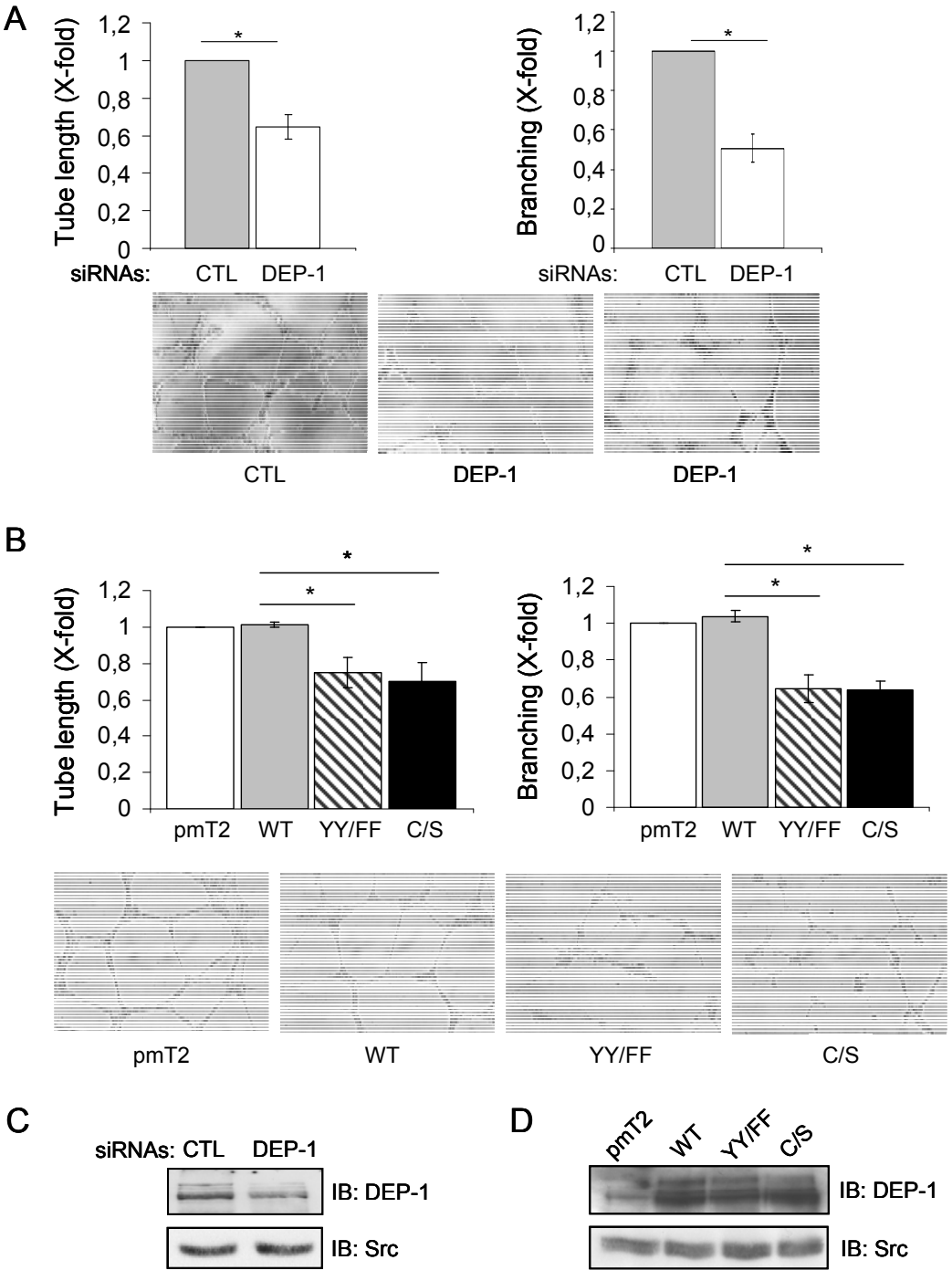

Figure 7: DEP-1, via Y1311 and Y1320, mediates the formation of branching

capillary-like structures.

(A) HUVECs transfected with CTL or DEP-1 siRNAs were plated 48h post-transfection on Matrigel (in duplicates) to promote the formation of capillary-like structures. The length of tubes formed as well as the number of branches at connecting nodes were quantified after 5-6h from 6 field/well. Results ± SD have been normalized using the average from the control condition and are representative of 3 independent experiments. *$p<0.05$ (B) HUVECS transfected with the indicated constructs were plated in duplicates on Matrigel to evaluate their ability to form branching capillary-like structures. Quantification was done as described above and is representative of 3 independent experiments. *$p<0.05$ (C) Representative DEP-1 expression level in RNAi-transfected cells. (D) Comparable expression level of DEP-1 and mutants in transfected HUVECs.

Discussion

Previous studies from our laboratory demonstrated that the knockdown of DEP-1 expression in endothelial cells impaired Src activation in response to VEGF and FGF (160). We further showed that this was a consequence of the defective dephosphorylation of the Src inhibitory Y529 in the VE-cadherin-associated fraction, which was identified as the major pool of activated Src in VEGF-stimulated endothelial cells (261). We now show that the VEGF-induced activation of Src is mediated through the phosphorylation of DEP-1 on the C-terminal Y1311 and Y1320, which allow its interaction with the Src SH2 domain and the dephosphorylation of the displaced inhibitory Y529. Consistent with the central role played by Src in angiogenesis, our studies further reveal the major function of DEP-1 and its C-terminus in the VEGF-dependent phosphorylation of VE-cadherin and Cortactin, (453, 458) and consequently, in the remodeling of intercellular junctions, permeability, cell invasion, and the formation of branching capillary-like structures. We thus propose that a phospho-displacement mechanism engaging DEP-1 pY1311 and pY1320 with the SH2 domain of Src is required for its activation in response to

VEGF stimulation, and that this is critical for the angiogenic response of endothelial cells.

The impaired capacity of DEP-1 Y/F mutants to associate with Src and promote its activation (Figures 2-4) reveals the importance of VEGF-dependent phosphorylation of DEP-1 as a mechanism controlling Src activation in this cell system. Supporting this conclusion, we found that the phosphorylation kinetics of DEP-1 overlapped with those of VEGF-induced Src Y418 phosphorylation (Figure 4). In addition, while phosphorylation of overexpressed WT DEP-1 on Y1320 was above that seen in control cells upon VEGF stimulation, no residual phosphorylation was detected in cells overexpressing the DEP-1 Y1311F/Y1320F mutant where Src activation was abrogated (Figure 4). On these bases, we then propose that the VEGF-dependent phosphorylation of Y1311/Y1320 controls the moment at which DEP-1 can interact with Src to dephosphorylate Y529, but without altering its ability to dephosphorylate other substrates not associating with DEP-1 via these residues. This mechanism is somewhat similar to what was reported for PTPε whereby tyrosine phosphorylation of the carboxy-terminal Y695 is essential for the specific dephosphorylation of Src on Y529, but not for the ability of PTPε to dephosphorylate other substrates (299). Importantly, we also demonstrated that the impaired ability of mutant DEP-1 to dephosphorylate Src was not due to its decreased catalytic activity, since it was as active as WT DEP-1 in a pNPP assay or following its co-expression with another substrate, VEGFR2 (Figure 3). Our results therefore identify growth factor-mediated phosphorylation of DEP-1 as another level of regulation of its functions, by determining both substrate specificity and kinetics of dephosphorylation.

We have shown in HEK 293T cells that DEP-1 is constitutively phosphorylated, and that this is dependent on the basal activity of SFKs (Figures 1, 3C). However, it is not clear yet how VEGF leads to this initial phosphorylation of DEP-1 in endothelial cells. As we have concluded from our work in HEK 293T cells, DEP-1 can auto-dephosphorylate or get dephosphorylated by other PTPs (Figure 1). Thus, it is tempting to speculate that a VEGF-induced event leading to the attenuation of PTP

catalytic activity could contribute to the increased phosphorylation of DEP-1. In that respect, reactive oxygen species (ROS), which have inhibitory effects on PTPs, might perhaps be involved (449). Coupled with a relatively high basal activity of Src in endothelial cells, the VEGF-induced increase in ROS (451) could lead to a decrease in DEP-1 activity, thus enabling the accumulation of phosphorylated DEP-1 and the consequent activation of Src. In agreement with this idea, it is interesting that ROS production in endothelial cells contributes to VEGF-induced Src activation and phosphorylation of VE-cadherin, and to the promotion of capillary formation and permeability (451, 459).

To further prove the contribution of DEP-1 Y1311/Y1320 to VEGF-mediated Src activation, we demonstrated that the phosphorylation of two well-known Src substrates, VE-cadherin and Cortactin, (450, 452) was abrogated in cells expressing the Y1311F/Y1320F, E1321Q or C/S mutants, similarly to what was observed in DEP-1-silenced cells (Figures 4-6 and Supplemental Figure 3). VE-cadherin tyrosine and serine phosphorylation is associated with the loosening of cell-cell junctions that promotes increased vessel permeability and the initiation of vessel sprouting and elongation (18, 460). In the case of Cortactin, its tyrosine phosphorylation promotes the local polymerization of branched actin that is important for stabilization of nascent membrane protrusions during cell migration and invasion (452, 456, 461). Its expression and tyrosine phosphorylation are also essential for VEGF-dependent migration (453, 455). In this context, the decreased phosphorylation of VE-cadherin and Cortactin, as well as the impaired localization of Cortactin at membrane protrusions, are highly consistent with the inability of DEP-1-silenced or mutant-expressing cells to remodel cell-cell junctions and regulate permeability in response to VEGF, or to invade and form a well-organized network of branching capillary-like structures (Figures 5-7). These data thus demonstrate for the first time a promoting role for endogenous and moderately overexpressed DEP-1 in the induction of pro-angiogenic functions of endothelial cells and also highlight the key role played by the C-terminal Y1311/Y1320. These findings also suggest that the defective vessel

remodeling and branching characterized in the DEP-1 mutant mouse (265) might partly be explained by impaired Src-dependent pathways. The reported localization of DEP-1 at endothelial cell-cell junctions (442) and its association with constituents of the VE-cadherin complex (160, 274, 275) strongly support its critical role in the activation of Src at this site (261) (Figures 4B, 5A). However, as DEP-1 also promotes cell-substratum adhesions, (318) its possible involvement to these sites as well is not excluded.

The results presented herein also importantly revealed that the expression level of DEP-1 is a critical factor dictating its substrate specificity and function. Moderate overexpression of DEP-1 increased Src activation associated with Y529 dephosphorylation, while higher levels also led to Y418 dephosphorylation and blocked VEGF-dependent tyrosine phosphorylation of VE-cadherin and endothelial cell invasion (Figures 3-4, Supplemental Figure 2). Thus, depending on DEP-1 expression levels, the dephosphorylation of Src might be inappropriate for the promotion of biological activities such as permeability and invasion. In that context, it is interesting to consider that similarly to the in vitro situation, the expression of DEP-1 in vivo is decreased in proliferating and migrating cells during vessel repair compared to adjacent quiescent endothelial cells (149, 262). This then suggests that DEP-1 might have bivalent functions, where moderate levels would promote VEGF-dependent Src activation in actively growing and invading endothelial cells, while its upregulated expression at confluence and the recruitment of VEGFR2 (269) would result in the downregulation of both Src and VEGFR2 activity (149, 160) to promote vessel quiescence and stabilization. Interestingly, a similar dual function of the receptor-like PTP CD45 was previously reported, where higher versus lower expression levels led to the differential dephosphorylation and activation of the SFK Lck, inducing both positive and negative regulatory functions in T cell signaling (462). In all cases, these regulatory mechanisms might be necessary to allow the downregulation of SFK signaling and the attenuation/termination of biological responses associated to cell quiescence.

In conclusion, these studies bring novel insights into the molecular mechanisms underlying the VEGF-dependent regulation of Src, which is a key promoter of angiogenesis and vascular permeability, and importantly recognize DEP-1 Y1311 and Y1320 as positive regulators of the angiogenic response.

Acknowledgments

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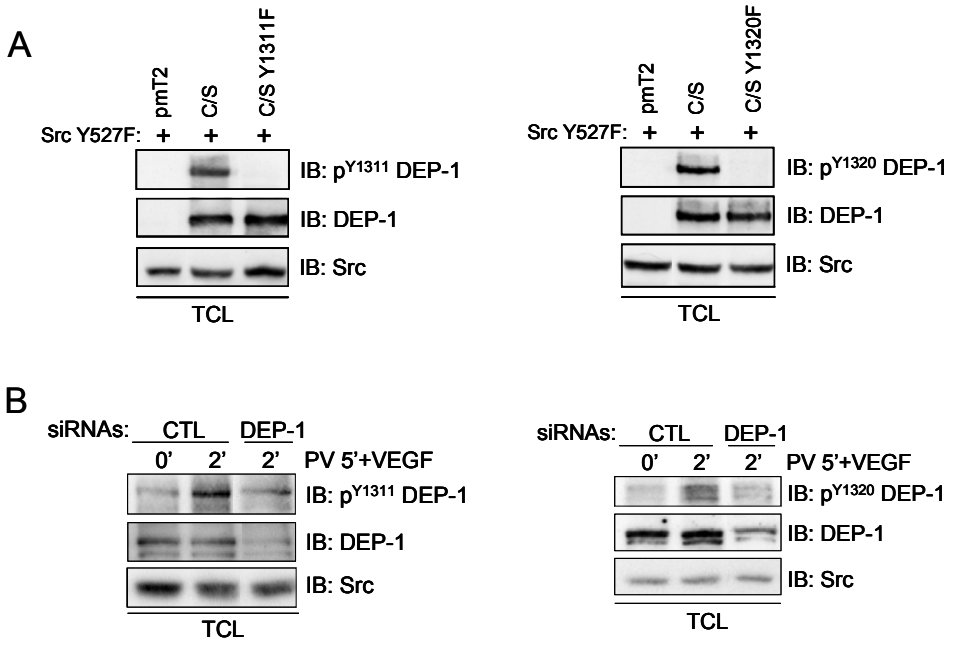
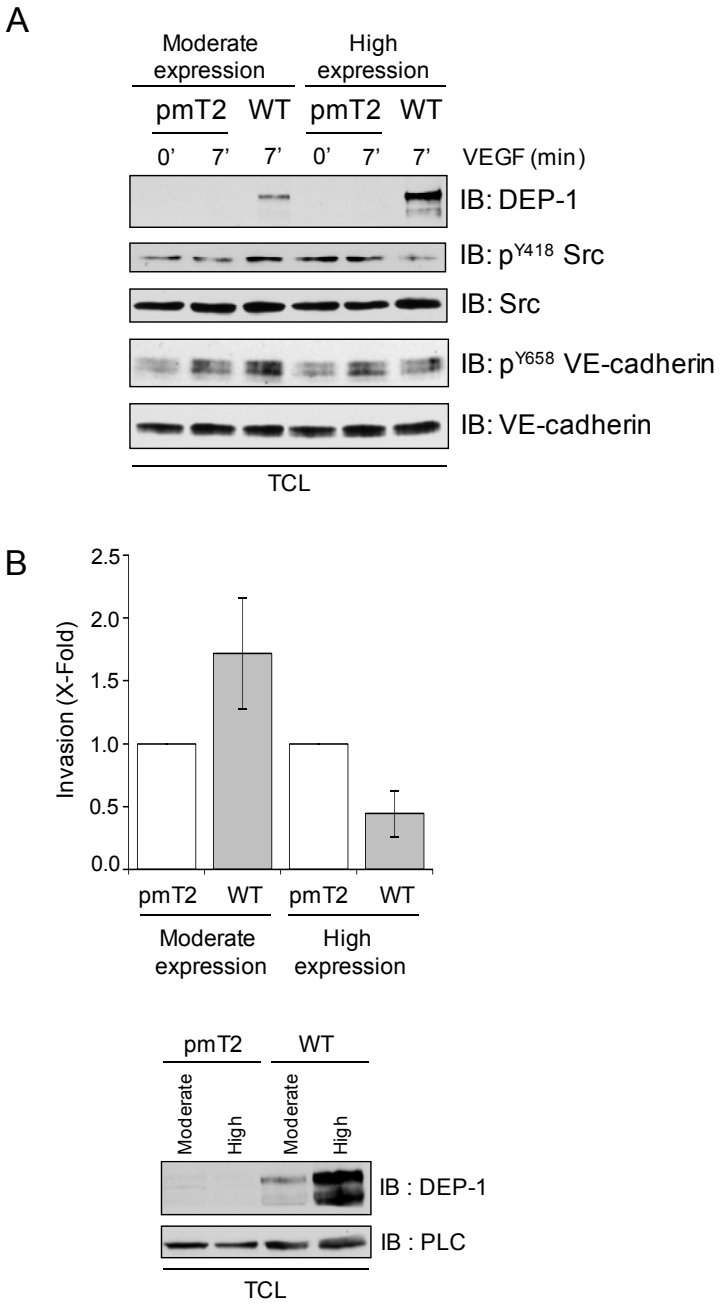
Supplemental Figures



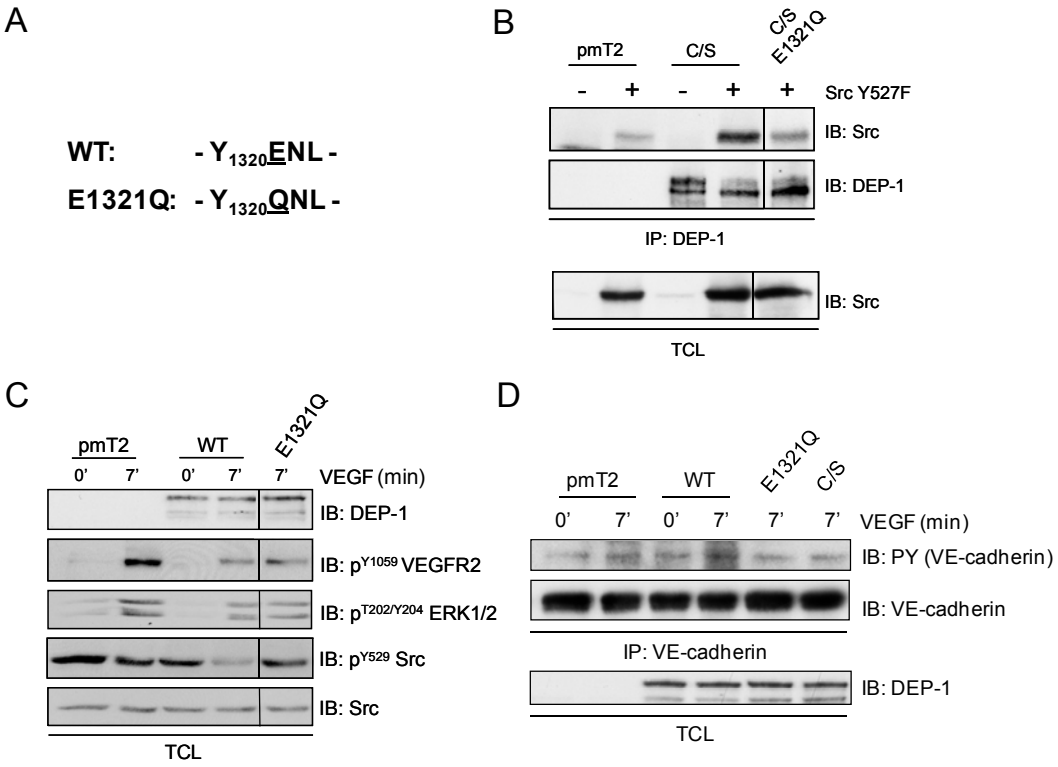
Supplemental Figure 1: Characterization of DEP-1 phosphospecific antibodies recognizing pY1311 and pY1320.

(A) HEK 293T cells were co-transfected with either empty vector (pmT2), DEP-1 C/S, C/S Y1311F or C/S Y1320F, with or without active Src (SrcY527F) to induce their tyrosine phosphorylation. Phosphorylated DEP-1 is detected in total cell lysates using phosphospecific antibodies recognizing pY1311 and pY1320 in cells expressing C/S DEP-1, but not when encompassing the Y1311F or Y1320F mutations, respectively. Immunodetection of DEP-1 and Src with the corresponding antibodies shows equal protein levels between conditions. (B) HUVECs were transfected with control (CTL) and DEP-1 siRNAs. Cells were serum-starved for 6h and incubated with pervanadate (100 μM) for 3 min before adding or not VEGF (50 ng/ml) for 2 min. Phosphorylation of endogenous DEP-1 is detected in VEGF-stimulated CTL cells with anti-DEP-1 pY1311 and pY1320, but not in DEP-1-silenced cells. Immunoblotting with DEP-1 and Src antibodies shows decreased expression of DEP-1 in the DEP-1 RNAi-transfected cells, but equivalent levels of Src, confirming constant protein loading.



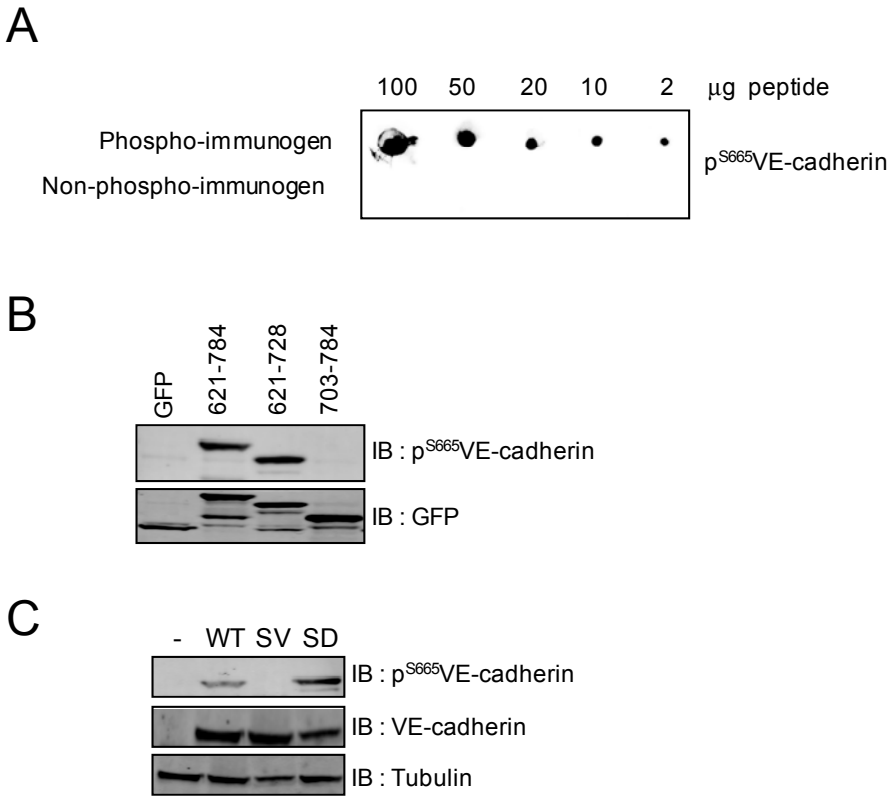
Supplemental Figure 2: VEGF-induced Src activation and downstream responses are differentially regulated by moderate versus high overexpression of DEP-1.

(A) BAECs were transfected with either 5 (moderate) or 15 (high) μg of empty vector (pmT2) or WT DEP-1. The activation status of Src as well as the phosphorylation of its substrate VE-cadherin were monitored by immunoblotting total cell lysates (TCL) with the indicated antibodies. Results are representative of 3 independent experiments. (B) BAECs were transfected with moderate versus high amounts or DEP-1 cDNA plasmids as described above. Their ability to invade Matrigel was performed as described in the Methods section. Lower panels, immunoblotting of DEP-1 shows moderate and high expression levels while PLCγ detection demonstrates equal protein loading. Results are representative of 2 independent experiments.



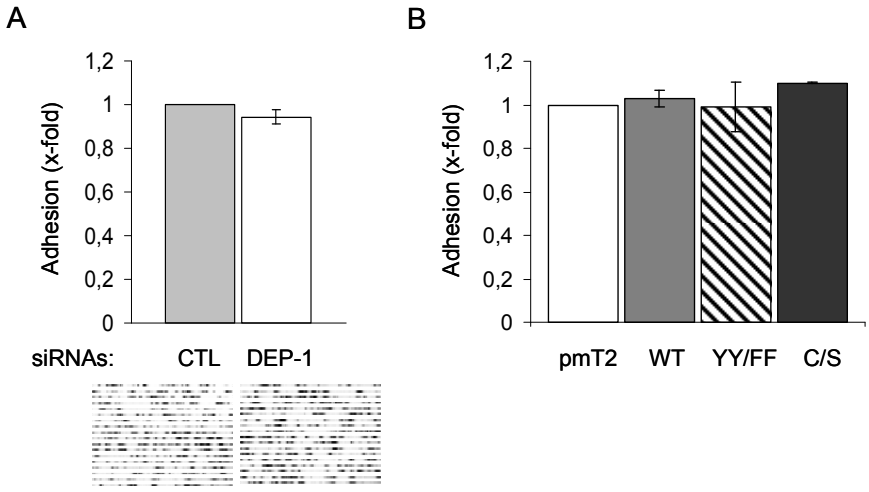
Supplemental Figure 3: Mutation of a Src consensus binding site downstream of Y1320 is sufficient to block optimal Src activation.

(A) The consensus Src binding site Y^{1320}ENL was mutated to Y^{1320}QNL to disrupt Src binding. (B) Empty vector (pmT2), DEP-1 CS and DEP-1 C/S E1321Q were expressed in HEK 293T cells, in the presence or not of active Src Y527F. DEP-1 was immunoprecipitated and the associated Src detected by immunoblotting with the Src GD11 antibody. Immunoblotting of total cell lysates with the Src GD11 antibody shows equal amounts of transfected Src. (C) BAECS were transfected with empty vector (pmT2), WT DEP-1 and the E1321Q mutant. Forty-eight hours later, serum-starved cells were stimulated with VEGF (50 ng/ml) for 7 min, and the activation status of Src was probed with the phosphospecific antibody detecting the inhibitory phosphorylation of Y529. Immunoblotting with phosphospecific antibodies detecting activated VEGFR2 and ERK1/2 shows that their level of phosphorylation are equivalent in cells expressing WT or the E1321Q mutant. (D) BAECs were treated as described in C. The phosphorylation of VE-cadherin was investigated following the immunoprecipitation of VE-cadherin and immunodetection with the PY99 antibody. All results are representative of at least 3 independent experiments.



Supplemental Figure 4: Specificity of p^{S665}VE-cadherin antibodies.

(A) Indicated amounts of the phosphorylated or non-phosphorylated VE-cadherin peptide (658-YDVSVLNSVRRGG-670) were immobilized on nitrocellulose membrane using dot-blot apparatus (Bio-Rad) and then blotted against p^{S665}VE-cadherin antibodies. (B) HEK-293T cells were transfected with cDNA constructs encompassing intracellular portions of VE-cadherin (amino acids 621-784, 621-728 and 703-784). GFP transfected cells serve as negative control. Lysates were processed for Western blotting using p^{S665}VE-cadherin and GFP antibodies. (C) Alternatively, cells were transfected with wild-type (WT), non phosphorylable (S665V) and phospho-mimetic (S665D) mutants of VE-cadherin. Cell lysates were processed as in (B).



Supplemental Figure 5: The expression level of DEP-1 has no influence on the ability of endothelial cells to adhere to Matrigel.

(A) HUVECs transfected with control (CTL) or DEP-1 siRNAs were plated in Matrigel-coated wells in duplicates and left to adhere in the presence of VEGF for 30 min. Adhesion was quantified by counting the cells remaining attached after staining with crystal violet (6 fields/well were counted). Results are representative of 3 independent experiments. (B) HUVECs transfected with empty vector (pmT2), WT DEP-1, DEP-1 Y1311F/Y1320F (YY/FF) or DEP-1 C/S were processed as described above. Results are representative of 3 independent experiments.

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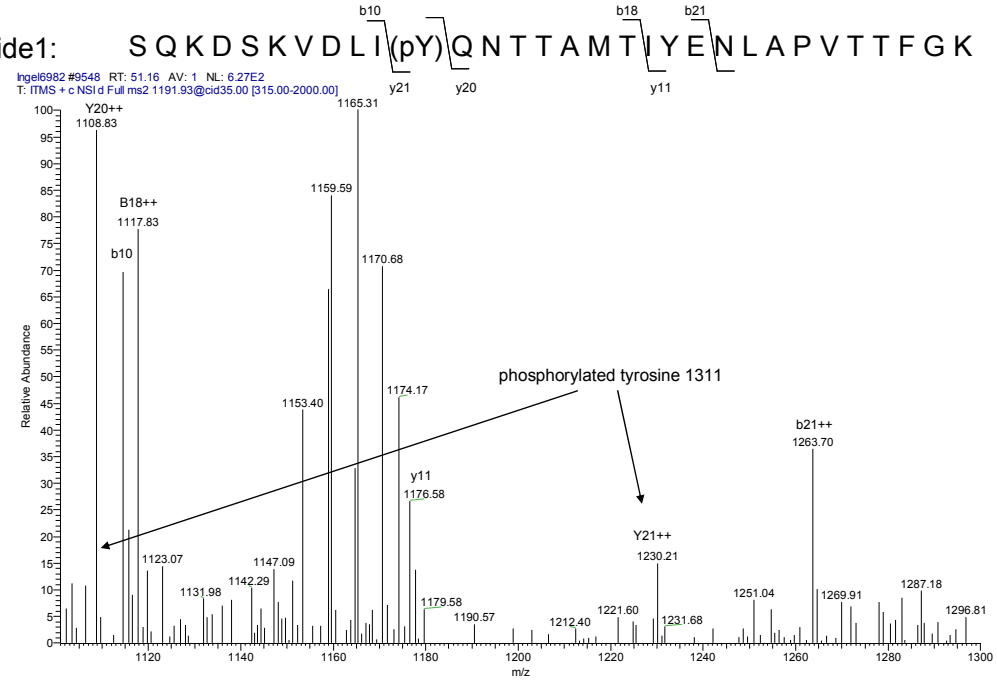
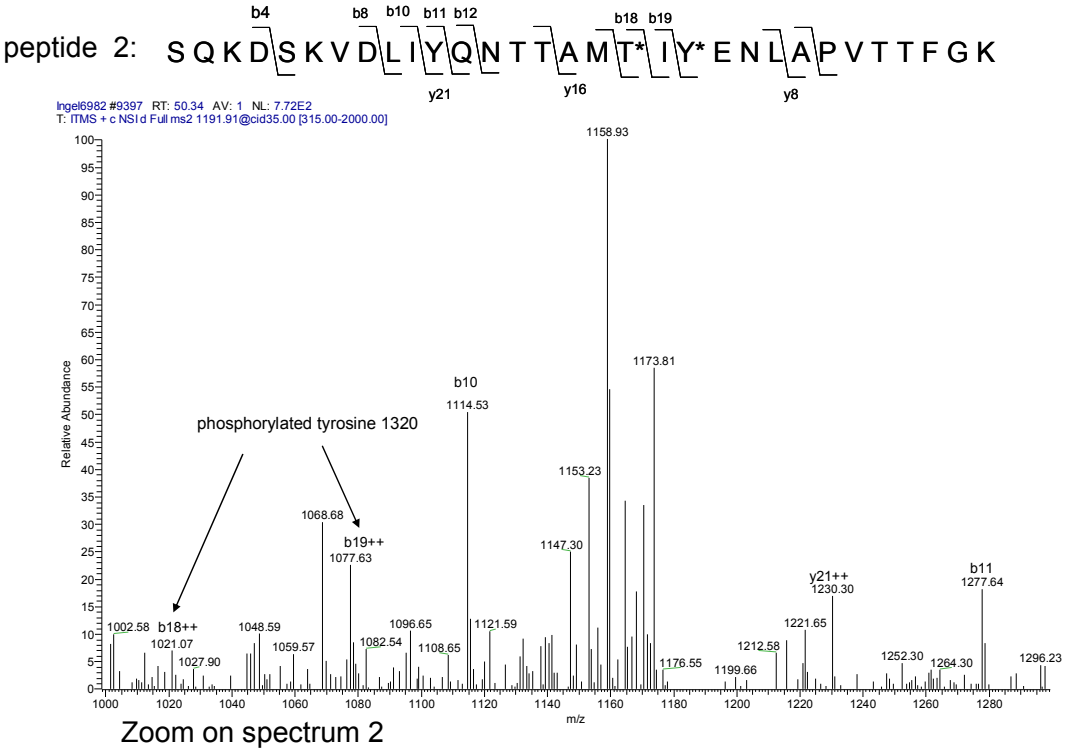


Zoom on spectrum1



Supplemental Figure 6: Analysis of phosphorylated DEP-1 D/A by mass spectrometry.

Myc-tagged Myr-DEP-1 D/A was immunoprecipitated with the Myc antibody from lysates of transfected HEK 293T cells. The Myr-DEP-1 D/A corresponding band was excised from the gel and subjected to in-gel trypsin digestion before analysis by electrospray mass spectrometry (ES MS/MS) at the Proteomics platform of the Centre génomique de Québec. Peptide sequences were determined using the software program MASCOT and validated using SCAFFOLD and ASCORE. The modification of 80 mass units to tyrosine as well as serine and threonine was included in the database searches to determine phosphopeptides. Following analysis, peaks could discriminate between phosphorylated tyrosine.

CHAPTER III

DEP-1 phosphorylation on threonine 1318 regulates its ability to promote Src activation, VE-cadherin phosphorylation and vascular permeability in endothelial cells.

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Author contribution

K.S. has done experiments in figures 1A and 1B, 3B, 4A and 4B, 5A, 5B and 5D. Figure 2C was done in collaboration with L.L. She wrote the paper with I.R.

S.L. has done experiments for figure 3A and 4C as well as 5C.

L .L. performed experiments in figure 1C, 2A and B. Figure 2C was done in collaboration with K.S.

I .R. has supervised the whole work and wrote this article with K.S.

Abstract

DEP-1 is a receptor-type protein tyrosine phosphatase with antiproliferative and tumor suppressive functions. Hence, several DEP-1 substrates include cell growth-promoting receptor tyrosine kinases and ERK1/2. However, we and others have also reported its ability to positively regulate Src family kinases and biological functions in endothelial and immune cells. We further demonstrated that upon VEGF stimulation of endothelial cells, phosphorylation of DEP-1 Y1311 and Y1320 in its C-terminal tail is required for the optimal activation of Src and promotion of crucial angiogenic cell functions via their interaction with the SH2 domain of Src. Here, we show that DEP-1 T1318 is a new phosphorylation site, important for the regulation of the Src pathway. Interestingly, we find that DEP-1 tyrosine phosphorylation is impaired when T1318 is mutated, suggesting that T1318 has a regulatory control over DEP-1 tyrosine phosphorylation. Consistent with these findings, DEP-1 T1318A has a decreased ability to bind and activate Src in HEK 293T cells. Similarly, the phosphorylation of both Y1311 and Y1320 is impaired upon VEGF stimulation of endothelial cells expressing DEP-1 T1318A, which is unable to promote the activation of Src and the phosphorylation of its substrate VE-cadherin. Consequently, VEGF-induced endothelial cell permeability is impaired when DEP-1 T1318A is overexpressed. As T1318 is in a CK2 consensus phosphorylation site, incubation of endothelial cells with the CK2 inhibitor TBCA or CK2 overexpression demonstrate that DEP-1 T1318 is phosphorylated in a CK2-dependent manner. Treatment of endothelial cells with TBCA also impedes VEGF-induced DEP-1 tyrosine phosphorylation and Src activation. Overall, our results support the conclusion that the CK2-dependent phosphorylation of DEP-1 T1318 regulates the tyrosine phosphorylation of its C-terminal tail. Phosphorylation of DEP-1 T1318 may thus work as a regulatory switch directing the activity of DEP-1 towards its substrate Src

in a spatio-temporal manner to allow optimal Src activation and the promotion of angiogenic endothelial cell responses.

Introduction

Angiogenesis, or the formation of new blood vessel from already existing vessels, is essential for life. Blood vessels constitute the first functional organ providing the organism with oxygen and nutrients during development. In adults, angiogenesis is implicated in the regulation of female reproductive functions and wound healing; however, it can also contribute to the development of various pathologies including rheumatoid arthritis and cancer (463-465). Thus, angiogenesis is defined as a hallmark of cancer, promoting tumor growth and metastases formation (58). Neovascularisation in normal and pathological conditions is driven by the ability of endothelial cells to respond to angiogenic factors. One of the most important angiogenic factor is VEGF, which mediates most biological functions of endothelial cells including vascular permeability, cell migration and capillary formation via its receptor VEGFR2 (108, 466). Tight coordination and regulation of VEGFR2 activity is essential to maintain appropriate VEGF-dependent signalling and cell functions. The regulation of VEGFR2 is mediated by several mechanisms including protein tyrosine phosphatases (PTPs) such as DEP-1/PTPRJ/CD148, which dephosphorylate VEGFR2 to attenuate or inhibit VEGF-induced signalling (149, 467).

The expression of the receptor-like PTP DEP-1 was initially reported to increase with cell density, suggesting that it was regulating cell contact-mediated growth inhibition (234). Consistent with these findings, DEP-1 expression was also demonstrated to promote breast cancer cell differentiation and to inhibit cancer cell proliferation (392, 394, 437, 468). Several reports of loss of heterozygozity of DEP-1 in human cancers further suggest a role for DEP-1 as a negative regulator of growth and as a tumor suppressor (396). Interestingly, increased endothelial cell proliferation and impaired vessel remodelling and branching were observed in DEP-1 knock-in mutant mice in which the catalytic domain and C-terminal tail of DEP-1 were substituted for GFP (265). As VEGFR2 is a major mediator of angiogenic responses,

these results suggested that DEP-1 was implicated in its regulation. Accordingly, in confluent endothelial cells, DEP-1 is found to associate with VE-cadherin and VEGFR2 at sites of cell-cell contact (adherens junctions) and to mediate contact inhibition via the dephosphorylation of VEGFR2 and inhibition of the MAPK/ERK pathway (149, 150, 469). Previous work in our laboratory demonstrated that DEP-1 dephosphorylates Y1054/1059 of VEGFR2 resulting in the global attenuation of VEGFR2 activity and consequently of major signalling pathways downstream of VEGFR2 (160). However, unexpectedly, DEP-1 also promotes the Src-Gab1-AKT pathway under these conditions via the dephosphorylation of VE-cadherin-associated Src at inhibitory Y529, thereby promoting Src activation and the induction of VEGF-mediated endothelial cell survival (160).

Phosphatases are usually regulated by a number of mechanisms to control their activity and to maintain appropriate cell signalling. Posttranslational modifications such as phosphorylation on tyrosine as well as serine/threonine residues were described to influence phosphatase activity (449). For instance, phosphorylation of CD45 on tyrosine increases its catalytic activity in T cells (202). Similarly, serine phosphorylation of PTPα was reported to increase its catalytic activity and also to regulate its substrate specificity towards Src (206, 470). DEP-1 tyrosine phosphorylation on tyrosine 1311 and 1320 was recently reported by our group (471). In VEGF-stimulated endothelial cells, the binding of Src to these residues results in the dephosphorylation of Src Y529 by DEP-1 and its activation. Src is implicated in the induction of a number of endothelial cell biological functions including permeability and angiogenesis (18, 114, 129, 158). The remodelling of adhesive endothelial cell contacts, which is essential for the induction of vascular permeability and sprouting angiogenesis, is mediated by the Src-dependent phosphorylation of VE-cadherins directly on Y658 and Y731 (327, 450). We recently demonstrated that via its phosphorylated Y1311 and Y1320 and its ability to regulate Src activation, DEP-1 regulates VE-cadherin phosphorylation, endothelial cell permeability, invasion and capillary tube formation in response to VEGF (471).

In this paper, we identify a novel regulatory phosphorylation site in the C-terminal tail of DEP-1. DEP-1 T1318 is essential for the proper tyrosine phosphorylation of DEP-1 and therefore modulates its ability to activate Src and downstream endothelial biological responses. We also find that CK2 is a potential kinase regulating T1318 phosphorylation and the activation of this crucial angiogenic pathway.

Materials and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECS, Cascade Biologics, purchased from Invitrogen were cultured (passages 1 to 4) on 0.2% gelatin-coated tissue culture dishes and maintained in M200 medium (Invitrogen) supplemented with LSGS and gentamycin 50μg/ml (Wisent). Bovine aortic endothelial cells (BAEC) and HEK 293T cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and gentamycin (50 μg/ml).

Reagents and antibodies

Recombinant human VEGF-A was obtained from the Biological Resources Branch Preclinical Repository of the National Cancer Institute Frederick Cancer Research and Development Center. 4-para-nitro-phenyl phosphate (pNPP) was from Sigma. Rat tail collagen type I was bought from Sigma. Anti-Src rabbit antibody (clone 36D10), p-Threonine antibody, pY658 VE-Cadherin, VE-cadherin, pY421 Cortactin and Cortactin antibodies were from Cell Signaling Technologies. Anti-pY^{418} Src rabbit antibody was from Invitrogen. Mouse anti-DEP-1 (143-41) and goat anti-DEP-1 antibodies were from R&D Technologies. Anti-DEP-1 (143-41) and PY99 anti-phospho-tyrosine mouse antibodies were from Santa Cruz Technology, and anti-Src (clone GD11) mouse antibodies were from Upstate/Millipore. Phosphospecific antibodies against DEP-1 pY1311 and pY1320 were generated by Genscript and have been described before (471). Antibodies to DEP-1 pT1318 were generated by GenScript against the CKDSKVDLIYQNTTAM[pT_{1318}]IYEN peptide. Secondary (HRP-conjugated) anti-rabbit and anti-mouse antibodies were from Cell Signaling and HRP-conjugated anti-goat antibody was from Santa Cruz.

cDNA constructs

Plasmids encoding human DEP-1 WT and C/S mutant (in pmT2) were kindly provided by Nicholas Tonks (Cold Spring Harbor Laboratory, NY, USA). The DEP-1 T1318A and DEP-1 C/S T1318A mutants were generated with the QuikChange XL site-directed mutagenesis kit (Stratagene). The Src Y527F plasmid was provided by Marc Prentki (CRCHUM, Montreal, Qc, Canada) and the CK2 construct with its corresponding empty vector pc/CMV by David Litchfield (University of Western Ontario, London, ON, Canada).

Cell transfection

3,5 x 10^4 cells/cm^2 of BAEC plated in 100 mm dishes were transfected with 6 µg of DEP-1, DEP-1 T1318A or C/S using Lipofectamine 2000 (ratio 1:2,5) following manufacturer's instructions (Invitrogen). Twenty-four hours post transfection, cells were starved O/N in serum-free DMEM. Stimulation was performed 48h post-transfection with VEGF (50 ng/ml). HEK 293T cells were seeded at 1,2 × 10^6 cells/10-cm dish and transfected 24 h later using the standard calcium phosphate method. Empty pmT2 vector or DEP-1 constructs (2 µg) were co-transfected with active Src (1 µg). Cells were lysed 48h following transfection. HUVECs were plated at 40 000 cells/cm2 and transfected 20h later with 8 µg of pmT2, DEP-1 WT and DEP-1 T1318A mutant using 20 µl of lipofectin (Invitrogen) in OptiMEM medium (Invitrogen), or 8 µg of CK2 and its corresponding empty vector per 60 mm-dish. For the CK2 experiment, medium was replaced the next day and cells were starved for 6h in M200 48h post-transfection. Cells were then stimulated with VEGF for the indicated time points and then lysed as mentioned below. Alternatively, transfected HUVECs were collected 6h after transfection and processed for the permeability assay.

Cell lysis, immunoprecipitation and Western blotting

Cells were solubilized in a 50mM Hepes pH 7,5 lysis buffer containing 0,5% Triton X-100, 0,5% Nonidet P40, 10% glycerol, 1mM EDTA, 150mM NaCl, 1mM phenylmethanesulfonyl fluoride (PMSF), 1mM sodium vanadate (Na3VO4), 5mM Sodium Fluoride (NaF), Aprotinin (10µg/ml) and Leupeptin (10µg/ml). For Src binding experiments in HEK 293T cells (Fig. 2A), DEP-1 was immunoprecipitated from cell lysates (200µg) with 2 µl of anti- DEP-1 antibody (R&D Systems) for 1,5h at 4°C, and further incubated for 1,5h at 4°C with 30 µl of a 40% Protein G-Sepharose bead suspension. Beads were then washed 3X with lysis buffer and immunoprecipitates, or total protein extracts (40-50 µg), were subjected to SDS-PAGE, and transferred onto Hybond-C Extra nitrocellulose membranes (Amersham Biosciences/GE Healthcare). Similarly, VE-cadherin was immunoprecipitated from BAEC cell lysates (200 µg) using 2 µl of antibody. Western blotting and antibody detection was carried out using appropriate HRP-conjugated secondary antibodies and chemioluminescence-based detection systems according to the manufacturer's recommendations (ECL; Amersham or Visualizer kit; Millipore).

Permeability assay

Transfected HUVECs (4 x 10^4 cells/cm^2) were seeded onto Transwell permeability inserts (6,5mm diameter, 1,0 µm pore size; BD Falcon, BD Biosciences) pre-coated with rat tail collagen type I (50µg/ml). After forty-six hours, the cells were serum-starved for 1h in M200 medium and then stimulated by adding VEGF (50ng/ml) with FITC-Dextran (40 kDa size, 1 mg/ml) in the upper chamber for 30 min. Permeability was determined by measuring the fluorescence at 520 nm (498 nm excitation) in 50 µl-medium aliquots taken from the bottom chambers, which were diluted with 200 µl of M200 medium. Fluorescence was detected with a Victor3 V fluorescence reader (Perkin-Elmer).

pNPP assay

DEP-1 immunoprecipitated from transfected HEK 293T cells was resuspended in 50 µl of a 50 mM HEPES [pH 7,4] assay buffer containing BSA (0,1 mg/ml) and DTT (3mM) and transferred into a 96-well plate. DEP-1 activity was tested against the phospho-peptide 4-para-nitro-phenyl phosphate (pNPP; 50 µl of a 50 mM stock in water), which was added to the wells and incubated 5-15 min at room temperature. The coloured product pNp was detected by readings at 405 nm using a Victor3 V fluorescence reader (PerkinElmer). pNPP hydrolysis after 5 min of reaction is reported in the graph of Fig. 2C.

VEGF stimulation of non-transfected HUVECs

HUVECs were plated at confluence (3 x 10^4 cells/cm^2) for 42h and starved for 6h. Cells were stimulated with VEGF 50 ng/ml for the indicated time points and lysed as described above.

Results

DEP-1 is phosphorylated on T1318 in the C-terminal tail.

Previous work in our laboratory identified Y1311 and Y1320 as major phosphorylation sites in the C-terminal tail of DEP-1 (471). Biochemical and biological characterization determined that these sites are critical for VEGF-mediated Src activation and the promotion of major angiogenic functions including the remodelling of cell-cell junctions and permeability, invasion and branching capillary formation (471). In an attempt to further identify novel regulatory sites, mass spectrometry analysis performed on constitutively tyrosine phosphorylated DEP-1 led to the identification of T1318 as a new phosphorylated residue (471). As this site is proximal to Y1320, we reasoned it might be involved in the regulation of the Src pathway and we began investigating its phosphorylation status in HEK 293T cells. As it was first discovered on tyrosine phosphorylated DEP-1, these investigations were carried out in cells co-transfected with active Src together with DEP-1 or DEP-1 T1318A encompassing the C/S mutation, which renders DEP-1 catalytically inactive and inapt to auto-dephosphorylate. Using a general anti-phosphothreonine antibody, Figure 1A shows that DEP-1 C/S is indeed phosphorylated in HEK 293T cells, while the DEP-1 C/S T1318A mutant shows reduced phosphorylation. Threonine phosphorylation of DEP-1, but not that of the T1318A mutant, is also revealed using a specific pT1318 DEP-1 antibody, further demonstrating that T1318 is a true phosphorylation site (Fig. 1B). To find out if T1318 phosphorylation had an impact on DEP-1 tyrosine phosphorylation in this cell system, total cell lysates of HEK 293T cells transfected with DEP-1 C/S or the T1318A mutant were immunoblotted with pY1311 and pY1320 specific DEP-1 antibodies. Figure 1C shows that in these conditions, the phosphorylation of proximal Y1320, but not that of Y1311, is

decreased when T1318 is mutated. These results thus demonstrate that DEP-1 T1318 is phosphorylated and can regulate the phosphorylation of Y1320 in HEK 293T cells.

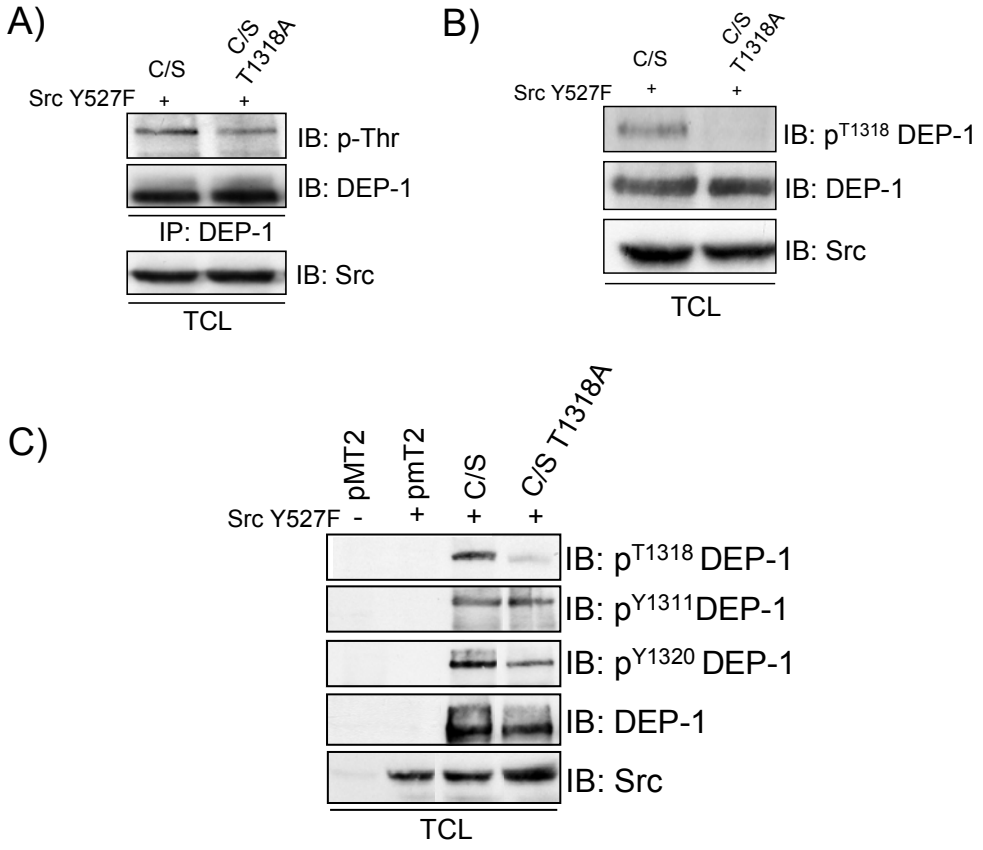

Figure 1: DEP-1 is phosphorylated on T1318 in the C-terminal tail.

(A) HEK293T cells co-transfected with active Src and either DEP-1 C/S or the C/S T1318A mutant (C/S = catalytically inactive mutant; unable to dephosphorylate itself). DEP-1 was immunoprecipitated with DEP-1 antibody and immunoblotted with a general p-Thr antibody. Immunoblotting with Src antibody reveals equal protein loading in both conditions. **(B)** HEK293T cells were transfected as in (A). Cell lysates were immunobloted with the pT1318 DEP-1 antibody. **(C)** Western blot analysis shows the DEP-1 tyrosine phosphorylation levels (PY1311 and PY1320) of pmT2, DEP-1 C/S or DEP-1 C/S T1318A mutant expressed in HEK293T cells. Cell lysates were immunoblotted with phosphospecific antibodies PY1311 and PY 1320 DEP-1.

Impaired ability of DEP-1 T1318A to bind and activate Src in HEK 293T cells.

Our previous studies demonstrated that Src binds via its SH2 domain to the phosphorylated Y1311 and Y1320 of DEP-1 (471). This binding releases the inhibitory interaction of Src pY529 from its own SH2 domain, and induces conformational changes that allow dephosphorylation of Y529 and increased phosphorylation of Y418 consistent with optimal Src activation (284). Since Y1320 phosphorylation seems to depend on T1318 phosphorylation (Fig. 1), we then next investigated the ability of DEP-1 T1318A mutant to bind and activate Src in HEK293T cells. Figure 2A shows that the DEP-1 T1318A mutant is impaired in its capacity to bind Src, as shown by immunoprecipitation experiments in transfected HEK 293T cells. This reduced Src binding results into decreased phosphorylation of the Src activatory site Y418 and higher phosphorylation of inhibitory Y529 in HEK 293T cells expressing DEP-1 T1318A compared to cells expressing WT DEP-1, consistent with decreased Src activity (Figure 2B). Similarly, catalytically inactive DEP-1 C/S is unable to dephosphorylate Src Y529 and activate Src (Figure 2B). Importantly, a phosphatase assay performed in the presence of pNPP as substrate revealed that the decreased ability of DEP-1 T1318A to activate Src is not due to its decreased catalytic activity (Figure 2C). The difference in PTP activity is not significant in vitro (p-value 0.2). These results demonstrate that the decreased activation of Src in HEK 293T cells expressing DEP-1 T1318 is due to the impaired tyrosine phosphorylation of Y1320 and decreased Src binding. Phosphorylation of T1318 is therefore required for DEP-1-mediated Src activation in HEK 293T cells.

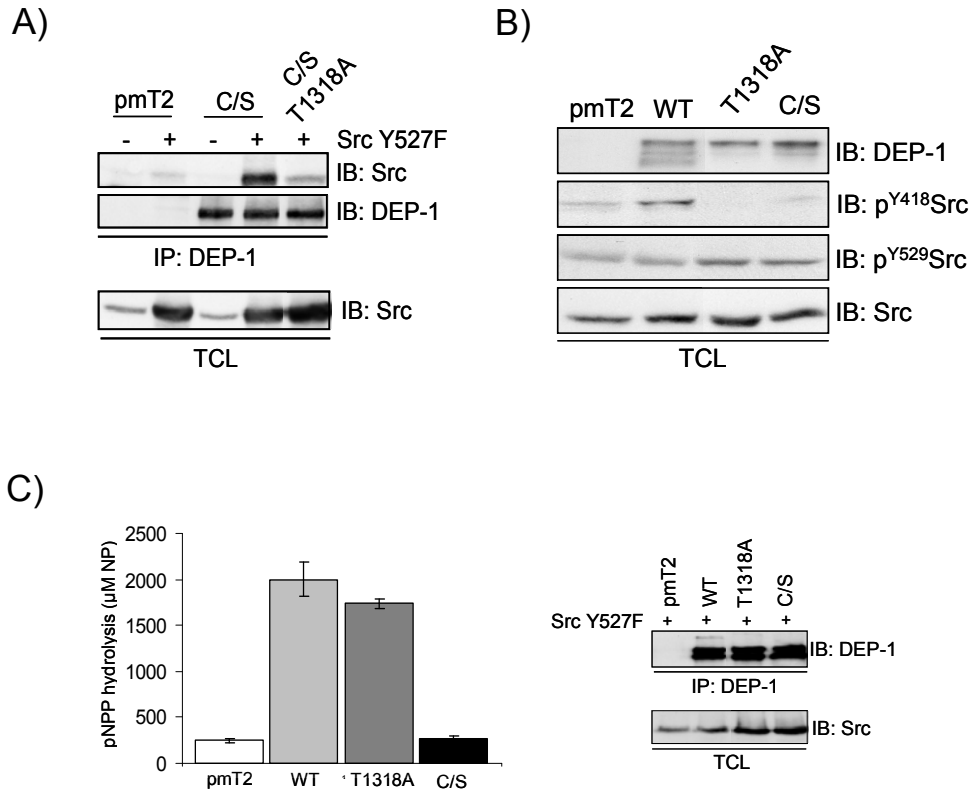

Figure 2: Impaired ability of DEP-1 T1318A to bind and activate Src in HEK 293T cells.

(A). Lysates of HEK293T cells co-transfected with DEP-1 C/S or the DEP-1 C/S T1318A mutant and constitutive active Src were immunoprecipitated with DEP-1 antibody. Association of DEP-1 with Src was detected by immunoblotting using Src antibody. **(B)** HEK 293T cells were transfected with DEP-1 WT, the WT T1318A mutant or DEP-1 C/S and Src phosphorylation was evaluated with a pY418 Src antibody. **(C)** Lysates of transfected HEK293T cells were immunopreciptated (IP) with DEP-1 antibody and IPs were used in a PTP activity assay. pNPP was used as a substrate to evaluate the PTP activity of DEP-1. Equal DEP-1 expression levels were determined by immunoblotting of immunoprecipitated DEP-1 using DEP-1 antibody. Results are representative of 3 independent experiments.

DEP-1 T1318 phosphorylation regulates VEGF-induced DEP-1 tyrosine phosphorylation and Src activation in endothelial cells.

To investigate the kinetics of endogenous DEP-1 T1318 phosphorylation in endothelial cells, HUVECs were grown to confluence and stimulated with VEGF

(Fig. 3A). T1318 phosphorylation of DEP-1 is observed in basal condition, and this is maintained or slightly induced until 1 minute upon VEGF stimulation, demonstrating that in these conditions, DEP-1 is constitutively phosphorylated on T1318 and that VEGF treatment rapidly downregulates the phosphorylation of this residue. In contrast, DEP-1 Y1311 and Y1320 phosphorylation is induced by VEGF treatment, as early as 30 sec post-stimulation, and reverts to basal levels after 7 min of stimulation. Interestingly, Src phosphorylation on tyrosine 418 is also rapidly induced after VEGF stimulation and overlaps with the induction of DEP-1 threonine and tyrosine phosphorylation (Fig. 3A).

To determine if DEP-1 T1318 phosphorylation has a regulatory impact on Y1311 and Y1320 phosphorylation in VEGF-stimulated endothelial cells, the phosphorylation status of these residues was investigated in HUVECs transfected with WT and T1318A DEP-1, or with empty vector (pmT2). Figure 3B shows that overexpression of WT DEP-1 results in higher levels of T1318 phosphorylation, especially before VEGF stimulation. Similarly, DEP-1 tyrosine phosphorylation is also increased compared to control (pmT2) cells, but mainly after VEGF stimulation. The elevated signal of DEP-1 phosphorylation on T1318 in DEP-1 T1318A mutant expressing cells could be due to higher DEP-1 expression levels in these cells compared to pmT2 expressing cells. However, in contrast to DEP-1 WT expressing cells, the phosphorylation of both Y1311 and Y1320 was reduced in these cells. Consistent with these results, Src phosphorylation on Y418 is impaired in VEGF-stimulated cells overexpressing DEP-1 T1318A compared to WT DEP-1-expressing cells (Fig. 4A, lower panel). These results thus suggest that T1318 phosphorylation promotes DEP-1 tyrosine phosphorylation and Src activation in VEGF-stimulated endothelial cells.

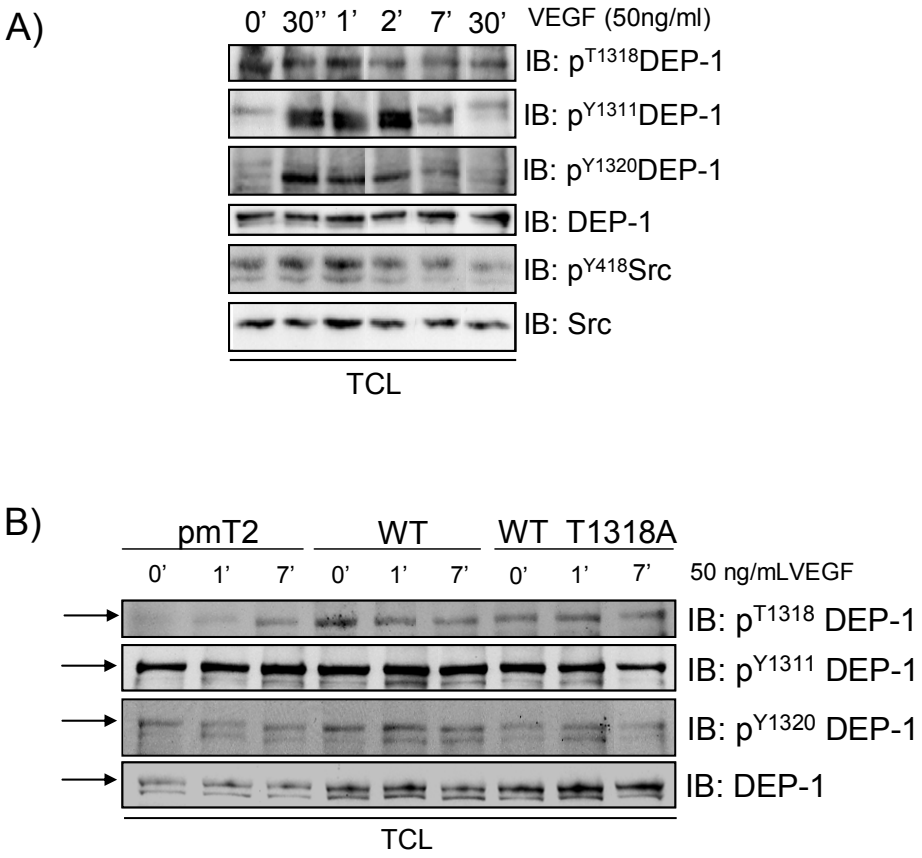

Figure 3: DEP-1 T1318 phosphorylation regulates VEGF-induced DEP-1 tyrosine phosphorylation and Src activation in endothelial cells.

(A) HUVECs plated at 3 x 10^4 cells/cm^2 for 42h were serum-starved for 6h and stimulated with 50 ng/ml VEGF for the given time points. Phosphorylation levels of DEP-1 phosphorylation were detected with the pY1311, pY1320 and pT1318 antibodies. Src phosphorylation was revealed using pY418 antibody. **(B)** HUVECs were plated at 4 x 10^4 cells/cm^2 and transfected with pmT2, DEP-1 WT or the DEP-1 T1318A mutant. Forty-two hours post transfection, cells were starved for 6h and stimulated with 50 ng/ml VEGF at given time points. Western blot analysis shows DEP-1 tyrosine phosphorylation levels of pY1311 and pY1320 and DEP-1 threonine phosphorylation levels of pT1318 in transfected cells. Arrows mark the upper band of DEP-1.

DEP-1 T1318 phosphorylation promotes the phosphorylation of VE-cadherin and the induction of endothelial cell permeability in response to VEGF.

The adhesion protein VE-cadherin is phosphorylated in a Src-dependent manner directly on Y658 and Y731 (327). Moreover, activation of a Src-Vav2-PAK pathway leads to the phosphorylation of serine 665 of VE-cadherin. Phosphorylation of VE-cadherin promotes its internalization and the remodelling of endothelial cell contacts (137). The VEGF-induced loosening of endothelial cell-cell contacts reduces the endothelial cell barrier function and increases vascular permeability (135, 332). Src has a prominent role in the mediation of vascular permeability and our previous studies demonstrated that DEP-1, via Y1311 and Y1320, is implicated in the remodelling of endothelial cell-cell junctions and the mediation of vascular permeability through the activation of Src (471). In this context, we investigated the impact of DEP-1 T1318 on these processes. BAECs were transfected with WT DEP-1, DEP-1 T1318A and the C/S mutants, as well as with empty vector, and then stimulated with VEGF. The tyrosine phosphorylation of VE-cadherin immunoprecipitated from WT DEP-1-expressing cells was strongly induced in response to VEGF stimulation, while this was completely blocked in cells expressing the DEP-1 T1318A mutant (Fig. 4A; upper panel). Moreover, Src-dependent VE-cadherin phosphorylation on Y658 was also decreased in HUVECs expressing the DEP-1 T1318A mutant compared to cells expressing WT DEP-1 (Fig. 4B). These results thus suggest that endothelial cells expressing the T1318A mutant maintain the integrity and rigidity of their intercellular contacts even in the presence of VEGF. DEP-1 T1318 phosphorylation would thus be required to induce the Src-dependent remodelling of endothelial cell-cell junctions. To test this hypothesis, we performed a vascular permeability assay with cells transfected with either the empty vector (pmT2), WT DEP-1 or the DEP-1 T1318A mutant. Figure 4C shows that permeability is induced in control cells following stimulation with VEGF, and that a similar induction is observed in cells overxpressing WT DEP-1. However,

permeability of the cell monolayer overexpressing the DEP-1 T1318A mutant is decreased below the basal level observed in non-stimulated control cells, suggesting that DEP-1 T138A mutant has a dominant negative function in this context. Altogether, these results demonstrate that DEP-1 T1318A is required to induce the remodelling of endothelial cell-cell junctions and endothelial cell permeability, suggesting a crucial role for DEP-1 T1318 in the appropriate regulation of endothelial barrier integrity in response to VEGF stimulation.

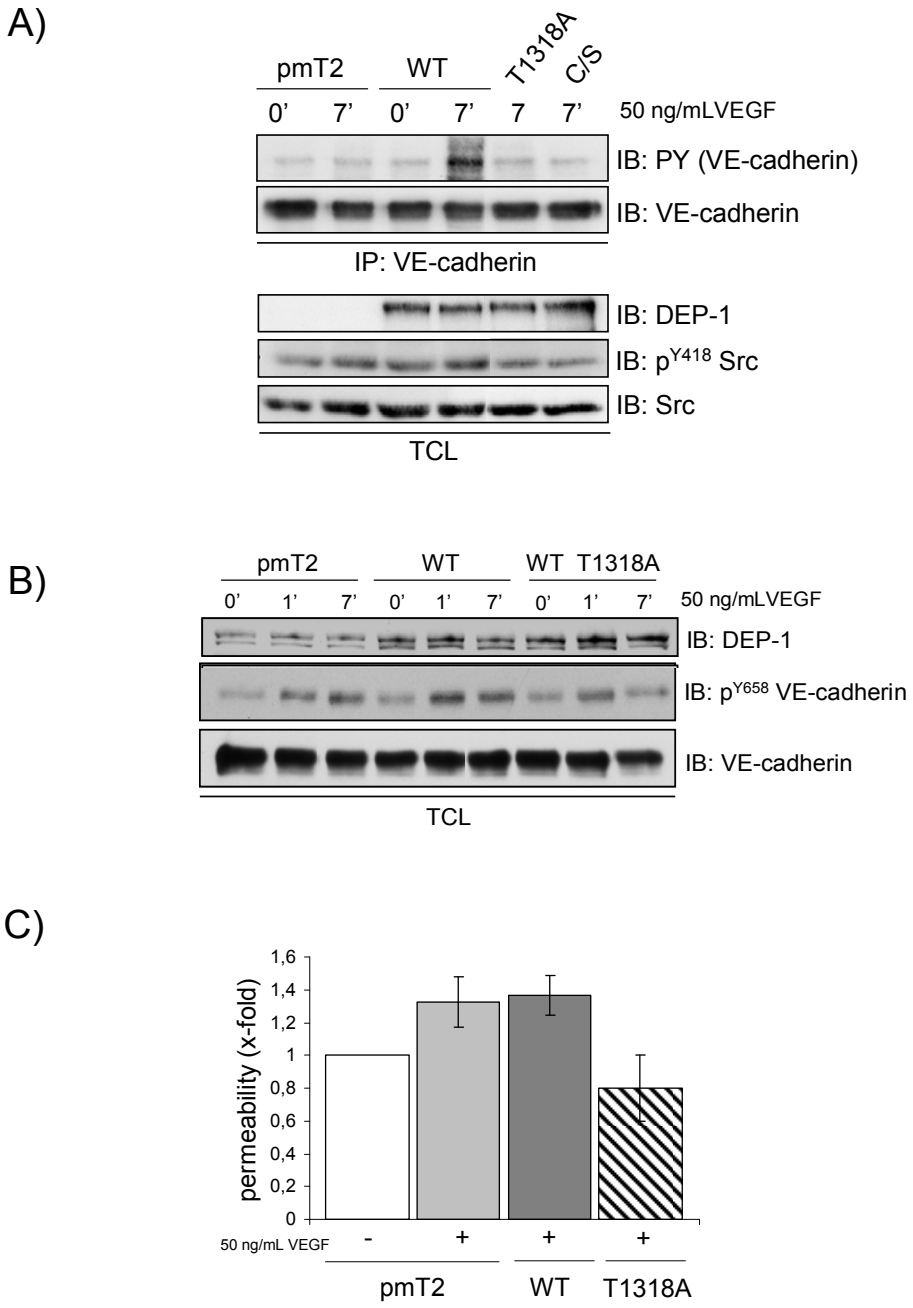

Figure 4: DEP-1 T1318 phosphorylation promotes phosphorylation of VE-cadherins and induction of endothelial cell permeability in response to VEGF.

(A) BAEC cells were plated at 3,8 x 10^4 cells/cm^2 and transfected with pmT2, DEP-1 WT, DEP-1 C/S or the DEP-1 T1318A mutant. Cells were serum-starved o/n and

for 7 minutes stimulated with VEGF (50 ng/ml). Total cell lysates were immunoblotted with the pY418-specific Src antibody to show activation levels of Src. The phosphorylation levels of VE-cadherin were determined following immunoprecipitation using a VE-cadherin antibody. Immunoprecipitates were immunoblotted with a general PY antibody. Results show that the DEP-1 T1318A mutant impairs VE-cadherin phosphorylation on tyrosine. **(B)** HUVECs were plated at 4 x 10^4 cells/cm^2 and transfected with pmT2, DEP-1 WT and DEP-1 T1318A mutant. 48h post transfection cells were starved and VEGF treatment induces Y1311, Y1320 and T1318 phosphorylation of DEP-1 concomitantly with phosphorylation of Y658 of VE-cadherin. **(C)** With pmT2, DEP-1 WT and DEP-1 T1318A transfected HUVECs (3 x 10^4 cells/cm^2, transfection for 6h) were submitted to a permeability test. Cells were plated on collagen type I coated inserts. After forty-six hours cells were starved for 1h and permeability was induced by 50 ng/ml VEGF. 50 µl aliquots were taken and diluted with 200 µl of M200 medium to measure the fluorescence level of the bottom chamber. Experiments were done in triplicate and means of relative permeability are shown.

DEP-1 T1318 is phosphorylated via CK2 in HEK392T and endothelial cells.

The T1318 sequence (T-X-Y-E) is a putative consensus sequence for the phosphorylation by the constitutively active serine/threonine kinase CK2 (T-X-X-D/E/pS/pY) (472, 473). Due to its implication in several cellular signalling events in response to diverse growth factors, CK2 can regulate a variety of biological functions (473, 474). Very interestingly, CK2 was reported to interact with the Cadherin complex, where DEP-1 co-localizes, and to regulate the integrity of cell-cell contacts (475, 476). In addition, CK2 inhibitors were also shown to inhibit the retinal angiogenic process (477). Therefore, we were interested in investigating the role of CK2 in DEP-1 threonine phosphorylation.

Treatment of transfected HEK 293T cells with TBCA (Tetrabromocinnamic acid), a potent specific CK2 inhibitor, resulted in the inhibition of DEP-1 threonine phosphorylation, as revealed with a general anti-phosphothreonine antibody (Fig. 5A). As showed before (Fig. 1B), the specific phosphorylation of DEP-1 on T1318 can be detected in control cells (vehicle-treated cells) expressing DEP-1 C/S, but not

in cells expressing the T1318A mutant (Fig. 5B). In these conditions, treatment of the cells with TBCA reduces the phosphorylation of DEP-1 on T1318 (Fig. 5B). Similar results were also observed following the incubation of endothelial cells with TBCA prior to VEGF stimulation (Fig. 5C). Indeed, compared to controls, DEP-1 T1318 phosphorylation was reduced in TBCA-treated endothelial cells. Densitometry analysis of DEP-1 T1318 western blot revealed that TBCA reduces basal and VEGF-induced DEP-1 T1318 phosphorylation levels in endothelial cells compared to the control cells (1.7 fold reduction of DEP-1 T1318 phosphorylation at basal levels and 1.5 fold or 2.4 fold reduction at one or seven minutes after VEGF, respectively). Concomitant with this reduction of DEP-1 T1318 phosphorylation, VEGF-induced Y1311 and Y1320 phosphorylation was also impaired. Accordingly, Src Y418 phosphorylation was greatly reduced in these conditions (Fig. 5 C). Thus, these results demonstrate that TBCA treatment decreases DEP-1 phosphorylation on T1318 concomitantly with decreased DEP-1 tyrosine phosphorylation and Src activation. The converse experiment, where CK2 or empty vector were transfected in endothelial cells, showed that CK2 increases the basal and VEGF-induced phosphorylation of DEP-1 T1318 (Fig. 5D). Higher T1318 phosphorylation correlated with elevated tyrosine phosphorylation, especially of Y1320, in CK2 overexpressing cells. Consequently, Src Y418 phosphorylation is increased along with phosphorylation of its substrate Cortactin in response to VEGF. Therefore, our results strongly suggest that CK2 is involved in the phosphorylation of DEP-1 T1318 in endothelial cells and regulates the DEP-1-dependent activation of the Src pathway in response to VEGF stimulation.

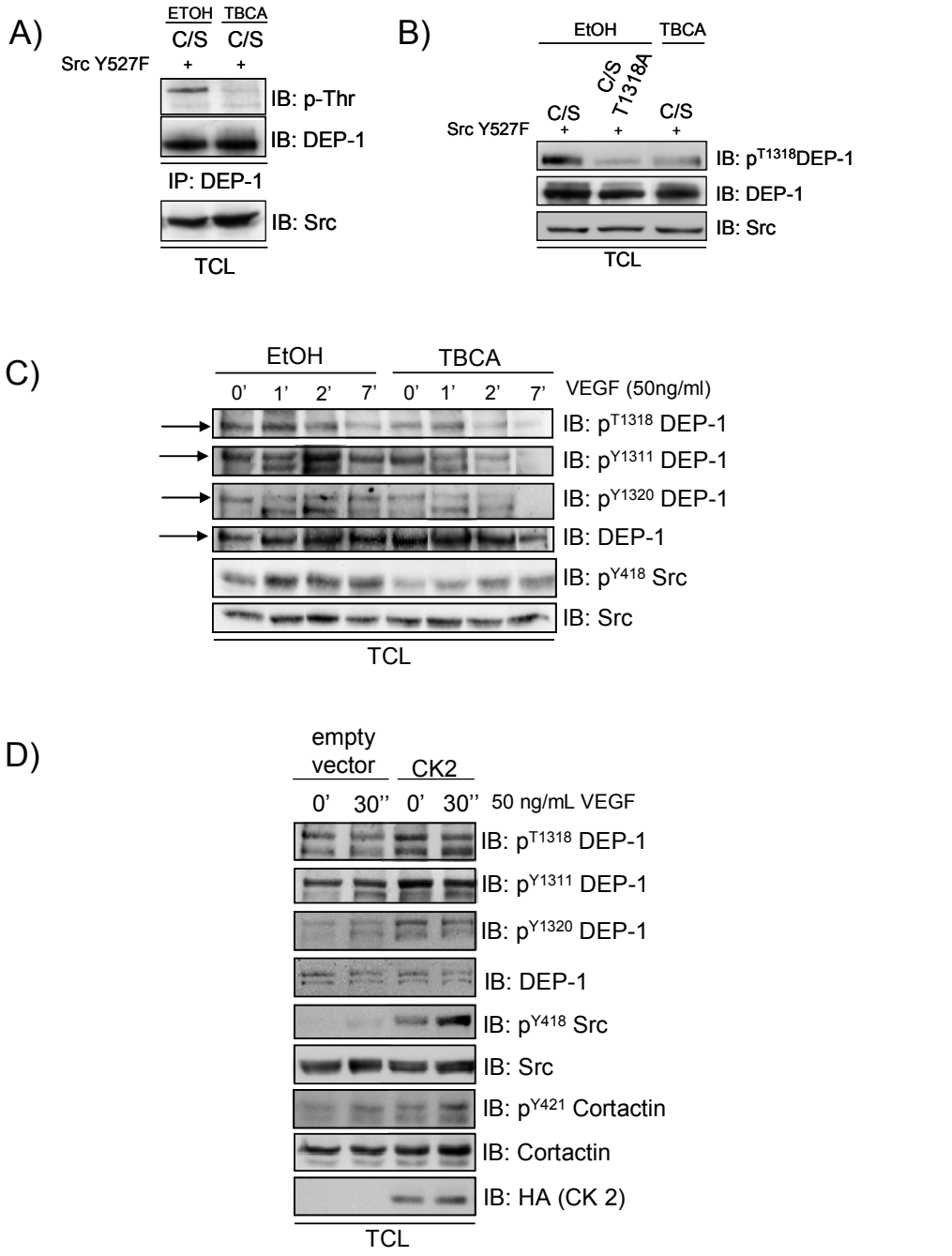

Figure 5: DEP-1 1318 is phosphorylated via CK2 in VEGF-stimulated endothelial cells.

(A) HEK293T cells transfected with DEP-1 were treated with 50 μM TBCA (Tetrabromocinnamic acid) or the empty vehicle (Ethanol) for 1h to inhibit CK2 activity. Cell lysates were immunobloted with pThr antibody. **(B)** HEK293T cells transfected with DEP-1 C/S or the C/S T1318A mutant were treated with 50 μM

TBCA or with the empty vehicle for 1h to inhibit CK2 activity. Cell lysates were immunobloted with DEP-1 pT1318 antibody. **(C)** HUVECs were plated at 3 x 10^4 cells/cm^2 for 42h, starved for 6h and treated at given time points with TBCA (50 μM) or empty vehicle for 1h. VEGF treatment induces T1318 phosphorylation of DEP-1 concomitantly with Src activation. Arrows mark the upper band of DEP-1. **(D)** HUVECs were plated at 3 x 10^4 cells/cm^2 and transfected with HA-tagged CK2 and the respective empty vector (pc/CMV). CK2 overexpression was determined with a HA-antibody. Phosphorylation levels of Src and its substrate Cortactin were determined with either pY418 Src or pY421 Cortactin antibody.

Discussion

In this study we identified a threonine residue in the C-terminal tail of DEP-1 that is localized proximal to Y1320. DEP-1 is phosphorylated in a CK2-dependent manner on T1318 in non-stimulated endothelial cells and this phosphorylation is rapidly decreased after VEGF treatment. Importantly, the phosphorylation status of DEP-1 T1318 overlaps with that of Y1311 and Y1320 in VEGF-stimulated cells, concomitantly with Src Y418 phosphorylation. DEP-1 T1318 phosphorylation promotes the transient VEGF-induced phosphorylation of Y1311 and Y1320, and is therefore critical for VEGF-mediated Src activation, VE-cadherin phosphorylation and endothelial cell permeability (Figure 6).

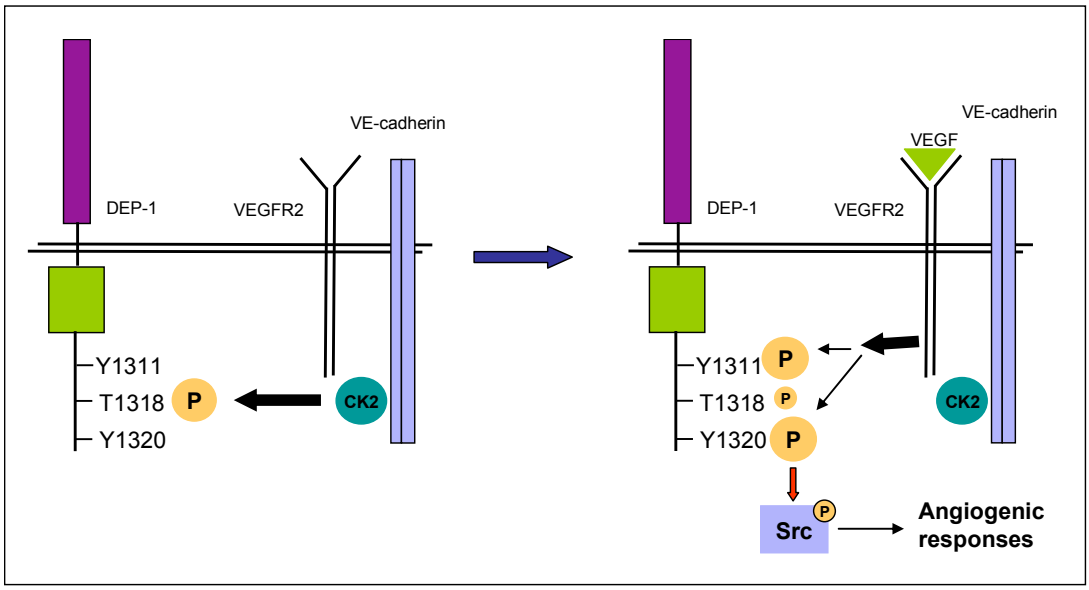

Figure 6: CK2-mediated DEP-1 T1318 phosphorylation and DEP-1 tyrosine phosphorylation in endothelial cells.

CK2 is translocated to cell-cell contacts proximal to the VEGFR2-DEP-1 complex. CK2 induces the phosphorylation of DEP-1 on threonine 1318 in endothelial cells. In response to VEGF, T1318 phosphorylation decreases slightly and Y1311 and Y1320 phosphorylation is induced concomitantly. DEP-1 T1318 phosphorylation increases the binding affinity of Src to DEP-1 Y1320 resulting in DEP-1-mediated dephosphorylation of the Src inhibitory Y529, resulting in Src activation. Activated Src promotes biological functions of endothelial cells.

This is the first report showing that DEP-1 threonine phosphorylation regulates its tyrosine phosphorylation. Given the consequences of this on Src activation and downstream signalling, this suggests that DEP-1 T1318 phosphorylation represents a novel regulatory mechanism directing DEP-1 substrate specificity towards Src with consequent Src activation in response to VEGF. Sequence analysis of phospho-mimicking mutations of T1318 (T1318E or T1318D) with Scansite software (http://scansite.mit.edu/) reveals that a negative charge at position 1318 would increase the affinity of Y1320 as a Src substrate (478). Thus, this suggests that the phosphorylation of T1318 might have direct impact on the phosphorylation of Y1320 by Src and perhaps by other Src-like kinases previously shown to phosphorylate DEP-1 (212, 471). This would lead to the increased binding of Src to Y1320, with consequent enhancement of Src activation.

We have observed that the T1318A mutation mainly affects Y1320 phosphorylation in HEK 293T cells expressing constitutively active Src. In contrast, the phosphorylation of both DEP-1 tyrosine residues is impaired by the mutation of DEP-1 T1318 in endothelial cells. We postulate that the decreased phosphorylation of Y1311 when T1318 is mutated could be a direct consequence of the decreased activation of Src in endothelial cells. Thus, by decreasing Src activation through decreased tyrosine phosphorylation of Y1320, T1318 would also indirectly affect the phosphorylation of Y1311 in endothelial cells.

Posttranslational modifications such as serine/threonine phosphorylation are known to regulate the activity or the substrate specificity of some phosphatases. Indeed, serine phosphorylation of PTPα was demonstrated to regulate its activity (470). It was

also suggested that the dephosphorylation of Ser204 in the juxtamembrane domain increases the binding affinity of Src to PTPα (206). Dephosphorylation of this residue is thus proposed to direct PTPα substrate specificity towards Src to promote its activation during mitosis (206). However, in the case of DEP-1, it is the increased phosphorylation of T1318 that directs its substrate specificity towards Src due to the increased ability of Src to bind tyrosine phosphorylated DEP-1 in these conditions. It is possible that DEP-1 phosphorylation on T1318 could influence the steric conformation of DEP-1. Based on our results, we show that DEP-1 phosphorylation on T1318 and Y1320 could be present at the same time early after VEGF stimulation. In this case, two negative charged phosphate groups would be in proximity to each other. Initially, we thought that the localization of two phosphate groups (PO_4^{2-}) leads to steric hindrance in the molecular protein structure due to their negative charges. The negative charge of DEP-1 T1318 phosphorylation could hinder the binding of a phosphate group to DEP-1Y1320 due to electrostatic repulsion between the two phosphate groups. Interestingly, scansite analysis of DEP-1 T1318E mutant (mutant which mimics phosphorylation on T1318) and our results of DEP-1 phosphorylation in endothelial cells provide opposed evidences.

The analysis of DEP-1 T1318E mutant revealed that this mutation improve the recognition of the consensus site for Src-mediated phosphorylation and Src binding in contrast to the uncharged threonine residue of DEP-1 WT. The better recognition of the consensus site by Src is due to the negative charged glutamatic acid mimicking the phosphorylated threonine 1318 (-ME_{1318}IYEN-). Interestingly, this mutation approximates the T1318 surrounding sequence closer to the optimal Src consensus sequence (–EEIYE-). Thus, the peptide sequence surrounding Y1320 has a higher affinity towards Src due to the phosphorylation on DEP-1 T1318 resulting in a better recognition of Y1320 by Src and/or a higher accessibility of Y1320 for Src-mediated phosphorylation.

The VEGF-dependent modulation of DEP-1 T1318 phosphorylation therefore represents a new regulatory mechanism controlling the intensity and kinetics of Src activation in VEGF-stimulated endothelial cells.

Based on the sequence surrounding T1318, we identified CK2 as a potential kinase. The multifunctional serine/threonine kinase CK2 is implicated similarly as Src in a wide range of signalling pathways downstream of various growth factor receptors (473). It is a tetrameric protein composed of two catalytic (α and α') and two regulatory subunits (β). Several studies proposed that CK2 is constitutively active, but the mechanism regulating this basal activity is ill-defined (472, 479). Of note, using inhibitors, CK2 was demonstrated to be involved in endothelial cell responses in a mouse model of proliferative oxygen-induced retinal neovascularization (477). CK2 was further described as a "master regulator" of angiogenesis due to its important role in the mediation of endothelial cell proliferation, survival, migration, tube formation and sprouting in vitro (480). Consistent with these positive roles for CK2 in angiogenic responses, we show here that CK2-mediated phosphorylation of DEP-1 T1318 is implicated in the promotion of Src-dependent endothelial cell functions such as endothelial cell permeability.

Interestingly, CK2 was reported to localize at adherens junctions where DEP-1 co-localizes (149, 274, 475). It was also shown to phosphorylate E-cadherin and lead to increased cell-cell adhesion (475, 476). Based on this, it is tempting to speculate that in order for VEGF to promote cell-cell junction loosening and endothelial cell permeability, CK2 activity would need to decrease or to dissociate from the VE-cadherin complex. Such scenarios would then be consistent with the detection of basal T1318 phosphorylation of DEP-1 in confluent unstimulated endothelial cells, and its rapid decrease upon VEGF stimulation. As CK2 localization is one way to regulate its activity on proximal substrates, its de-localization from VE-cadherin complexes could represent one mechanism to regulate its access to DEP-1, which co-localizes to these structures (481, 482). Alternatively, as CK2 was shown to be inactivated by ROS in a mouse model of cardiac hypertrophy (483), the localized

production of ROS at intercellular junctions upon VEGF stimulation could also be involved in the fast decline of CK2 activity at this site (484, 485).

In conclusion, we show herein that DEP-1 phosphorylation on T1318 is mediated by CK2 and promotes VEGF-induced DEP-1 phosphorylation on tyrosine, Src activation and endothelial cell functions. Thus, we report for the first time that DEP-1 T1318 phosphorylation displays a regulatory mechanism over DEP-1 tyrosine phosphorylation and DEP-1 mediated cell signalling.

Acknowledgements

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CHAPTER IV

Novel role for the protein tyrosine-phosphatase DEP-1/PTPRJ as a promoter of breast cancer cell migration and invasion.

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Author contribution

K.S. has done experiments for figure 1B in collaboration with L.L. Further K.S. performed experiments for figure 3, 4, 5, 6 and 7. She worked in collaboration with L.M. in the tissue microarray experiment. She wrote the paper with I.R.

L .L. performed experiments for figure 1A, B and figure 2.

L.M. worked in collaboration with K.S. on the tissue microarray experiment for figure 8.

A-M. M-M. provided the tissue microarray and gave helpful comments to the experimental design of the tissue microarray study.

I .R. has supervised the whole work and she wrote this article with K.S.

Abstract

Tight control of protein tyrosine kinase-dependent activities by protein tyrosine phosphatases (PTP) is essential for the induction of proper biological responses. DEP-1 is a PTP known for its antiproliferative and tumour suppressive functions. Indeed, many of its identified substrates are growth factor receptors, and it is frequently deleted and/or mutated in several human cancers including that of the breast. We have previously shown that DEP-1 plays an essential role in Src activation and the mediation of pro-angiogenic functions in endothelial cells. Since Src activation is also critical for breast cancer cell invasion and metastasis, we investigated if DEP-1 expression was related to the activation of the Src in breast cancer cells. We show here that DEP-1 expression is higher in the invasive MDA-MB 231, HS578T and BT-549 basal-like breast cancer cell lines compared to the less invasive MCF7, T47D and SK-BR3 luminal-like breast cancer cell lines. We demonstrate that the knockdown of DEP-1 decreases Src phosphorylation on Y418 in the basal-like cells, but not in luminal-like cell lines. Phosphorylation of the Src substrates Cortactin and FAK, which are associated with pro-invasive signalling, is also decreased in DEP-1-silenced MDA-MB 231 and HS578T cells. Consistent with these results, DEP-1 mediates the migration of these cell lines and the proper localization of Src, Cortactin and Actin at the leading edge of migrating cells. Furthermore, DEP-1 promotes the invasion of MDA-MB 231 and HS578T cells into Matrigel, but not that of T47D and SK-BR3 cells. In addition, we find that DEP-1 levels are further elevated in a MDA-MB 231 metastatic derivative cell line compared to the parental cell line, and that this correlates with higher phosphorylation of SrcY418, Cortactin and FAK, as well as increased invasion. Importantly, immunohistochemistry analysis of 141 breast tumour samples reveals that DEP-1 expression correlates with the decreased overall survival of patients and anterior breast cancer history. Collectively, our findings support the novel idea that

DEP-1 can act as a promoter of breast cancer progression, via Src activation and the induction of an invasive cell program.

Introduction

Protein-tyrosine phosphatases (PTP) are important antagonists of receptor tyrosine kinases (RTK) in cell signalling events. The expression of most tumour suppressive PTPs is altered in cancer, due to epigenetic and/or genomic modifications including mutations, deletions or amplifications (405). In particular, DEP-1/PTPRJ/CD148 is a PTP that has been reported to be mutated or deleted in several human cancers including those of the breast, colon, lung and thyroid cancer (396, 398). In addition, SNPs (single nucleotide polymorphisms) found in the extracellular domain of DEP-1 were associated with thyroid carcinomas, the risk to develop colorectal cancer, breast cancer and childhood ALL, suggesting a prominent role of DEP-1 in tumorigenesis (486, 487). Recently, DEP-1 was also shown to suppress EGFR-induced proliferation by limiting endocytosis of activated EGFR, supporting the idea that DEP-1 has a tumour suppressive role (213).

Breast cancer is the most frequently diagnosed cancer in women and most breast cancer deaths are due to the formation of metastases at distant organs, in preference into the bone, the lung and the brain (488-490) . Based on gene expression profiling two groups of breast cancer can be defined: Luminal-like breast cancer is reported to be Estrogen receptor positive (ER+) and represents the largest number of breast cancer cases. In contrast, basal-like breast cancer is ER- and represents only 15% of breast cancer (362, 363, 368). This subtype of breast cancer is generally more aggressive and highly express genes associated with proliferation, suppression of apoptosis, migration and invasion (365, 366, 491). Consistently, patients affected by the basal-like type of breast cancer have a poor clinical outcome.

Src and the other members of the Src family kinases (SFK) represent the largest group of non-receptor membrane-associated kinases (492) . Further, Src can promote many cellular processes such as proliferation, migration/invasion and survival of cancer cells (373, 375, 493, 494). Its activity is mainly regulated by the

phosphorylation level of the inhibitory tyrosine residue Y530 (in human). This residue is highly phosphorylated and bound intra-molecularly to Src's SH2 domain when Src is inactive and in a closed conformation. Dephosphorylation of Y530 leads to Src activation which is often accompanied by autophosphorylation of tyrosine 419 in the activation loop of Src. Based on its central role in several cell signalling pathways, Src can induce pro-invasive pathways that promote cancer progression (495). It is often over expressed or overactivated in many human cancers including that of the breast (340, 495-497). It is known as an essential player in survival and establishment of bone metastases mediating osteoclast activation and bone resorption (360).

One of the well-characterized Src substrates associated with the migratory and invasive capacity of cancer cells is Cortactin. Cortactin has regulatory and scaffolding roles in cytoskeletal organization by stabilizing Actin structures, but also in the regulation of membrane trafficking (498-500). Stabilized Actin supports the formation of protrusion and lamellipodia at the leading edge of migrating cells where Cortactin was shown to localize. FAK is another Src substrate contributing to the pro-invasive phenotype of cancer cells (501, 502). FAK resides at focal adhesion and in some instances at cell adherens junctions, where it is involved in the promotion of cell motility, via Actin assembly, and the formation of lamellipodia (503-506).

Here, we show that DEP-1 promotes Src activation, migration and invasion of basal-like breast cancer cells, which in contrast to luminal-like breast cancer cell lines, express higher levels of DEP-1. Importantly, consistent with a role in cancer progression, the expression of DEP-1 is found to be further increased in a derivative metastatic of basal-like cells in vitro, and correlates with reduced overall survival in breast cancer patients and the occurrence of an anterior breast cancer history. In conclusion, we identify DEP-1 as a novel and essential player in the promotion of pro-invasive signalling in basal-like breast cancer cell lines and as a potential predictor of the risk to develop metastases.

Material and methods

Cell culture and reagents

Breast cancer cells were cultured in DMEM, supplemented with 10% FBS and 50 µg/ml Gentamycin. Primary Antibodies for anti-pY421 Cortactin, anti-pY418 Src, anti-pY861 FAK, FAK (Invitrogen), anti-Cortactin, clone 4F11 (Millipore), anti-DEP-1 (R&D), Src, clone 36D10 (Cell signalling) were used for Western blots. Anti-PTPRJ Prestige Antibody (Sigma) was used for immunohistochemistry.

siRNA and cDNA Transfection and cell stimulation

For siRNA transfection experiments MDA-MB 231 and HS578T cells were plated at 2 x 10^4 cells/cm² SK-RBR3 and T47D cells were plated at 3 x 10^4 cells/cm². Cells were transfected 20h post-plating with DEP-1 (Hs_PTPRJ_3_HP) or Allstars control siRNAs (Qiagen) at a final concentration 25 nM using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. For cDNA overexpression, cells were plated at 2,2 x 10^4 cells/cm² and transfected with 1,2 µg of cDNA/35 mm and 5 µl of Lipofectamine 2000. Twenty-four hours post-transfection, cells were starved O/N in serum-free medium and stimulated 40h post-transfection with 5% FBS. Cells were lysed in a 50mM Hepes pH 7,5 lysis buffer containing 0,5% Triton X-100, 0,5% Nonidet P40, 10% glycerol, 1mM EDTA, 150mM NaCl, 1mM phenylmethanesulfonyl fluoride (PMSF), 1mM sodium vanadate (Na3VO4), 5mM Sodium Fluoride (NaF), Aprotinin 10µg/ml and Leupeptin 10µg/ml. Cells were lysed, subjected to SDS-PAGE and immunoblotted using standard protocols (160).

Invasion assay

MDA-MB 231 and MDA-MB 231 1833 variant cells were plated at 5 x 10^3 cells/cm^2; HS 578Tcells were plated at 2 x 10^4 cells/cm²; and SK-BR3 and T47D cells at 3 x 10^4 cells/cm². siRNA were transfected as mentioned above. For overexpression

experiments, MDA-MB 231 cells were plated at 2,2 x 10^4 cells/cm² and transfected as described above. Forty hours post-transfection, cells were trypsinized and centrifuged at 1000 rpm for 5 min. Cells (1 x 10^4) were seeded on a polycarbonate membrane (Costar Transwell Corning, pores 8µm) pre-coated with 50 µl of Matrigel (BD) at a final concentration of 3,8 mg/ml (MDA-MB 231, siRNA experiments), 3,7 mg/ml (HS578T), 2 mg/ml (T47D) and 1 mg/ml (SK-BR3) for 2 h at 37° C (siRNA experiments), and 50 µl of Matrigel (3,55 mg/ml) for MDA-MB 231 cells overxpressing DEP-1 cDNA. Cells were allowed to invade the Matrigel for 16-18 h. Cells were then fixed with formalin for 20 min and stained O/N with Crystal violet (1,2% in 20% methanol). Stained cells on the lower side of the polycarbonate membrane (cells that invaded the Matrigel and crossed the membrane) were counted. Experiments were done in duplicate.

Scratch wound healing assay

MDA-MB 231 and HS 578T cells were plated at 5 x 10^4 cells/cm² and 2 x 10^4 cells/cm² respectively and transfected as described in the above section. Medium was replaced after 16h and cells were incubated until they reached 100% confluence. Forty hours post-transfection, the cell monolayer was scratched with a yellow tip to create 3 lines. Photos were taken immediately after scratching at 2 microscope fields/scratch (at t=0). Cells were allowed to migrate for 22h and when the wound was closed at 80-90%, cells were fixed with formalin and stained O/N with crystal violet. Images of the microscope fields photographed at t=0 were taken at t=22h. Wound closures were quantified using Image Pro Plus Software (Version 5.1; MediaCybernetics, Bethesda, MD) and Microsoft Excel. Gap width at t0 and tfinal were taken and the ratio t0/tfinal calculated in control and DEP-1-depleted cells. The gap width ratios of control cells were compared to those of DEP-1 knockdown cells.

Immunofluorescence

MDA-MB 231 were plated at 2 x 10^4 cells/cm² and transfected with siRNA as described above. Twenty-four hours post-transfection, cells were plated on glass coverslips (2 x10^5 cells/2cm²) and grown for 2 days. The cell monolayer was scratched and cells were allowed to migrate for 6h before fixation in Formalin-buffered solution for 20 min. Slides were washed three times with PBS and permeabilized for 5 min with TritonX-100 (0,25% in PBS). Slides were washed three times with PBS and then blocked with FBS (8% in PBS) for 30 min. Antibodies detecting DEP-1 (clone 143-41 R&D, 1:50), Src (clone 36D10, 1:100), Cortactin (clone 4F11, Millipore 1:100), and Phalloidin-AlexaFluor 594 (1:150) were used to stain proteins of interest. Secondary antibodies were diluted 1:800 in FBS (8% in PBS). Slides were mounted with ProLong Gold antifade reagent containing DAPI (Invitrogen/Molecular Probes). The quantitative analysis of the membrane localization of Src, Cortactin and Actin was performed in 6 microscopic fields at 40 x magnification. Cells with localization of Src, Cortactin and Actin at the membrane were counted in control cells and the number compared to cells treated with DEP-1 siRNA. Experiments were done in triplicate and in every experiment 100-200 cells were evaluated in every experiment.

Tissue array and immunohistochemistry (IHC)

TMAs were constructed as previously described (Svotelis et al., 2011). Immunohistochemical studies were performed on representative tumor areas annotated by a pathologist. The tissue microarray contains a total of 141 cases of breast cancer. The original data set was filtered to eliminate incompletely stained cores (5 of 141). The samples were stained with Prestige DEP-1 antibody (Sigma) diluted 1:20 and incubated for 60 minutes at 37^{o}C. Immunohistochemistry was performed using the Ventana Benchmark XT automated immuno-stainer (Ventana Medical Systems, Tucson, AZ, USA). We used the ultraView Universal DAB detection kit (#760-500) with 30 minutes antigen retrieval in cell conditioning

buffer#2. TMA slides were scanned on the VS-110 (Olympus Corporation, Japan), using the VS-ASW FL 2.4 software (Olympus, Germany).

Three independent observers evaluated DEP-1 expression levels in the TMA. The immunohistochemical data were transformed into positive (1) and negative (0) staining for DEP-1.

Statsitical analysis

Survival curves were calculated according to the Kaplan Meier method coupled with a log-rank test for survival analysis. Overall survival was computed from the date of diagnosed primary tumour to the date of death or the last follow up. The correlation of clinical data with DEP-1 expression was done with Pearson/Spearman correlation. All statistical analysis were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Higher DEP-1 expression in basal-like breast tumor cell lines mediates Src activation.

DEP-1 has been characterized as an inhibitor of cell proliferation and a tumor suppressor in many cancer types (234, 392-394, 437). However, despite these negative regulatory roles, we previously demonstrated that DEP-1 could also positively modulate Src activity and biological functions such as invasion in endothelial cells (160, 471). Since Src activity is associated with breast cancer progression and increased invasiveness, we thus wondered about the expression status of DEP-1 in breast cancer cell lines and if it was involved in the regulation of the Src pathway (493, 495).

As a starting point to test this possibility, we evaluated the expression level of DEP-1 in a series of human breast cancer cell lines known for their high invasive character (basal-like cell lines; MDA-MB 231, HS578T and BT549), or displaying low invasiveness (luminal-like cell lines; SK-BR3, T47D and MCF-7). Surprisingly, we observed higher DEP-1 expression levels in basal-like cell lines compared to luminal-like cell lines under the same culture conditions (Fig. 1A). To test if DEP-1 expression in highly invasive cell lines correlates with Src phosphorylation on tyrosine 418, DEP-1 expression was knocked-down by RNAi transfection. Results shown in figure 1B demonstrate that Src tyrosine 418 phosphorylation is decreased in DEP-1-silenced basal-like cell lines, whereas no changes or slightly induced phosphorylation are observed in lower invasive cell lines. These findings demonstrate that highly invasive basal-like cell lines express higher levels of DEP-1, which are critical for the optimal phosphorylation/activation of Src.

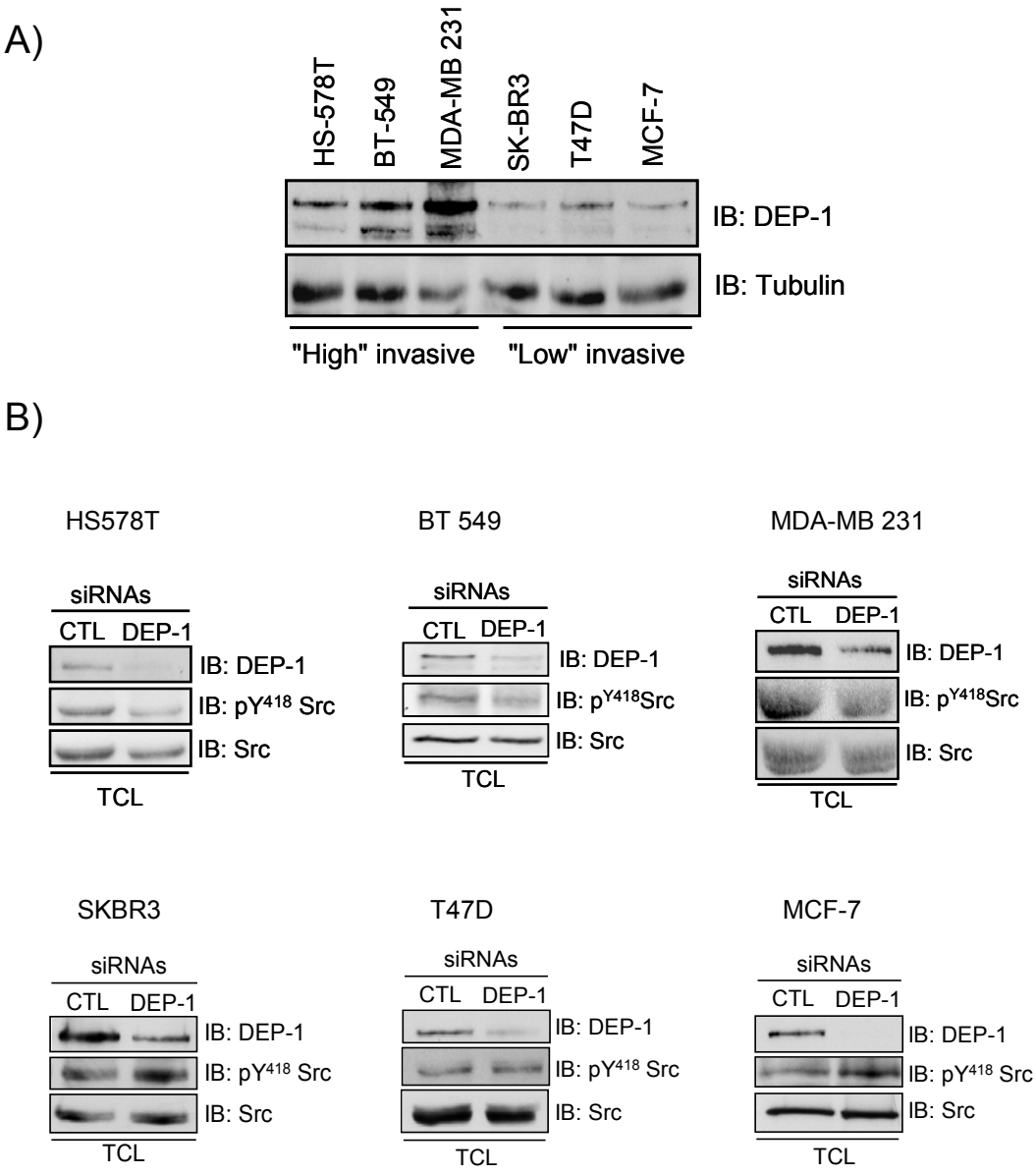


Figure 1: Higher DEP-1 expression in basal-like breast tumour cell lines mediates Src activation.

(A) Breast tumour cell lysates (50 µg) were immunoblotted with DEP-1 antibody. Immunoblotting with Tubulin antibody was used for loading control. (**B)** All breast cancer cell lines tested in A) for DEP-1 expression were transfected with control or DEP-1 siRNA and cell lysates were immunoblotted with DEP-1 and pY418 Src antibodies. Results representative for 2-4 independent experiments.

DEP-1 promotes Src activation and pro-invasive signaling in MDA-MB 231 and HS578T cells.

Activated Src can induce a pro-invasive signaling pathway in cancer cells, via the phosphorylation of its downstream targets Cortactin and FAK (498, 502, 505, 507). To find out if DEP-1 expression was regulating this pathway, DEP-1 was depleted in the MDA-MB 231 and HS578T basal-like cells. As shown in Figure 1B, Src phosphorylation on Y418 is decreased in the basal condition and following FBS stimulation (Fig. 2A, B). Consistent with this result, the phosphorylation of Cortactin on the Src-dependent tyrosine residue 421 and of FAK on tyrosine 861 were similarly impaired in DEP-1-depleted cells compared to the control (CTL) MDA-MB 231 and HS578T cells. Altogether, these results demonstrate that in invasive breast cancer cells, DEP-1 regulates Src activation and the activation of Src-dependent downstream pathways associated with a pro-invasive phenotype.

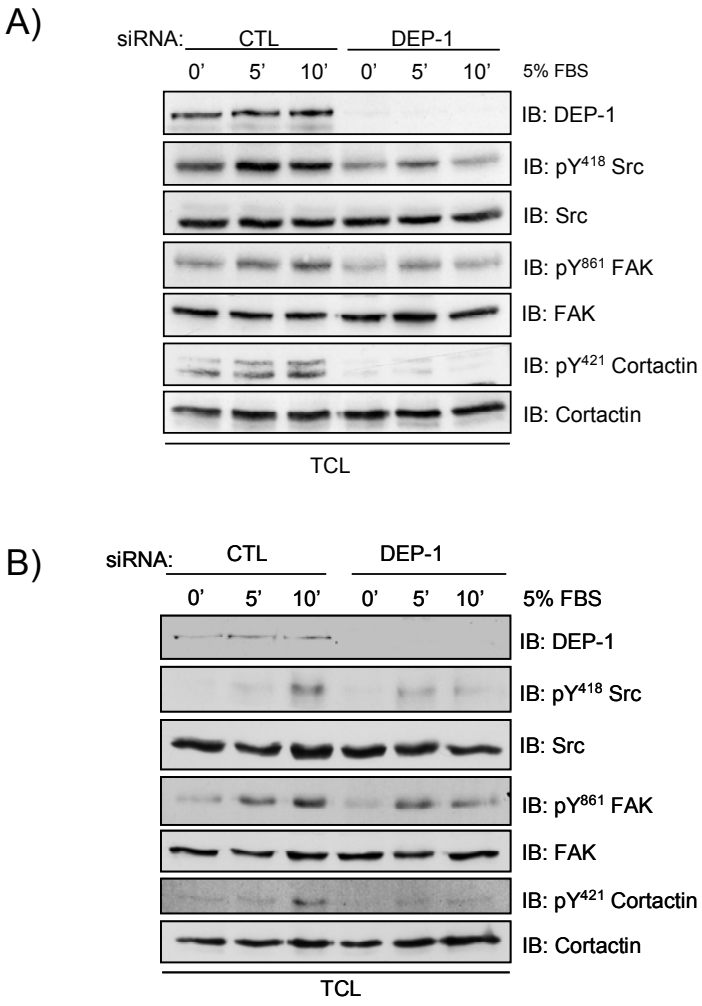


Figure 2: DEP-1 promotes Src activation and pro-invasive signaling in MDA-MB 231 and HS578T cells.
(A) MDA-MB 231 cells transfected with control (CTL) or DEP-1 siRNAs, serum-starved O/N and stimulated with FBS (5%) at given time points. Immunoblotting with Src Y418, Cortactin pY421 and FAK pY861 antibodies determined the phosphorylation level of Src, Cortactin and FAK. DEP-1 silencing correlates with inhibition of Src, Cortactin and FAK phosphorylation. (**B)** HS578T cells were treated as mentioned in (A) and stimulated with 5% FBS at given time points. Phosphorylation levels of Src, Cortactin and FAK were determined as in A). Results are representative for 4 individual experiments.

DEP-1 promotes the migration of invasive breast cancer cells and regulates the localization of Src and Cortactin and the Actin organization at the leading edge of migrating cells.

Cell migration and invasion are key steps in metastases formation and consequently in cancer progression. Several signalling pathways lead to the Src-dependent activation of Cortactin and FAK and consequently to increased cell migration/invasion (498, 502, 505). As we find that DEP-1 is required for the Src-dependent phosphorylation/activation of Cortactin and FAK (Fig. 2), we next investigated the implication of DEP-1 in the mediation of breast cancer cell migration in a scratch assay. Figure 3 shows that closure of the gap was delayed In DEP-1-depleted MDA-MB 231 and HS578T cells compared to their corresponding controls, suggesting that DEP-1 is required for the appropriate migration of these invasive breast cancer cell lines. Cell counts of DEP-1-depleted or control cells were similar 24 h post transfection, suggesting that inhibition of proliferation could not be inferred as a cause of delayed wound closure of DEP-1-depleted cells (Supplemental Figure 1).

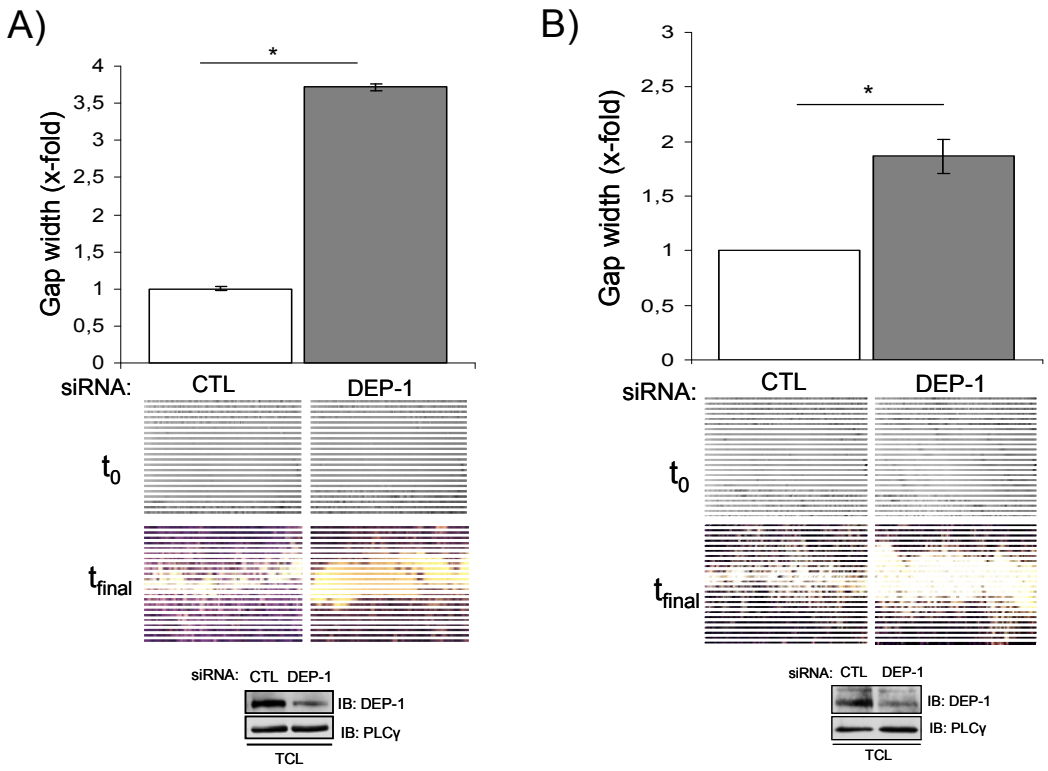


Figure 3: DEP-1 promotes the migration of invasive breast cancer cells.

(A) MDA-MB 231 or **(B)** HS578T cells transfected with control (CTL) or DEP-1 siRNAs. Cell monolayers were scratched 40h after transfection and cells were allowed to close the gap for 20h. Cells were then fixed and stained with crystal violet. 2 photos per gap were taken immediately after the scratch (t0) and the end point of gap closure after 20h (tfinal). Gap width at t0 and tfinal were measured with ImageProPlus and the gap width calculated t0/tfinal. The gap width ratio of control cells were compared to DEP-1 knockdown cells. Immunoblots show the level of DEP-1 silencing in both cell lines and PLCγ was used to show equal protein loading. Results shows the average of 4 experiments, * $p< 0.05$.

Cortactin promotes the appropriate arrangement of the cytoskeleton resulting in directed cell movement (499). Since we observed a defect in cell migration and Cortactin phosphorylation/activation, we next investigated if Src and Cortactin were properly localized at the leading edge of migrating MDA-MB 231 cells. Interestingly, we found that DEP-1 is enriched at the membrane of the migrating front (Fig. 4A, C).

This is consistent with reports that DEP-1 is localized at membrane ruffles in macrophages (508). Further, we demonstrate that Src, Cortactin and Actin are also enriched at the membrane of the front migrating cells (Fig.4 A-D). Conversely, Actin is about 30% less organized at the membrane in DEP-1-depleted cells (Fig. 4B, D). In addition, the enrichment of Src and Cortactin is also decreased by about 50% at this site in DEP-1-depleted cells (Fig. 4 A-D). These results suggest that the presence of DEP-1 at the leading edge of moving cells is essential for the appropriate localization of Src and Cortactin to promote directed and coordinated cell migration.

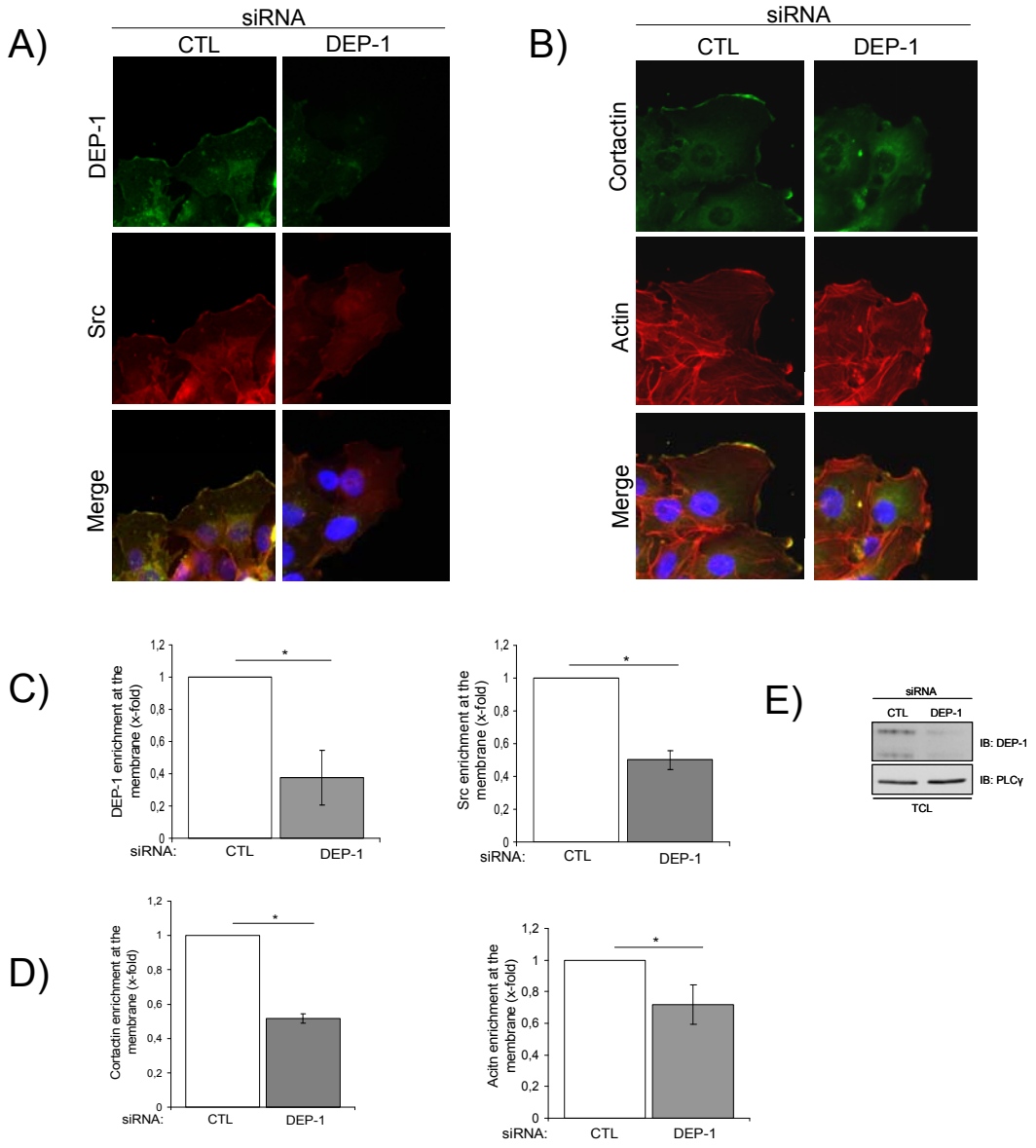


Figure 4: DEP-1 regulates the localization of Src, Cortactin and the arrangement of Actin at the leading edge of migrating cells.
(A), (B) MDA-MB 231 were transfected with CTL or DEP-1 siRNA. Cells were plated 24h later on glass cover slips. The cell monolayer was scratched 42h after plating on the coverslips. Cells were allowed to migrate for 6h into the gap. Cells were fixed after 6h, stained with anti-DEP-1 and anti-Src antibodies (A) or anti-Cortactin and Phalloidine-Alexa Fluor594 (B). DAPI staining was used to mark the cell nucleus. **(C), (D)** Quantification of at the leading edge localized DEP-1, Cortactin, Src and Actin in wound-edge cells were evaluated in control and DEP-1

siRNA cells. **(E)** Western blot shows inhibition of DEP-1 expression in MDA-MB 231 cells. Results shown are means ± SEM of cells which localized these proteins at the leading edge and are representative for 3 independent experiments. p-values were calculated with Mann-Withney U-test * $p < 0.05$

DEP-1 promotes invasion of basal-like breast cancer cells.

Since breast cancer cell invasion is a process dependent on Src and the ability of cells to move, we next asked if invasion was also affected in DEP-1-depleted basal-like cells. Fig. 5A and B show that DEP-1-silencing strongly decreased the invading capacity of MDA-MB 231 and HS578T cell lines into Matrigel. However, the invasive capacity of DEP-1-depleted luminal-like breast cancer cell lines (SK-BR3, T47D) was similar to control cells, or was slightly increased in the case of DEP-1-silenced SK-BR3 cells (Fig. 5C, D). Altogether, these results show that DEP-1 promotes invasion of the basal-like cell lines MDA-MB 231 and HS578T, but has no effect or slightly suppresses invasion in the luminal-like cell lines T47D and SK-BR3. Collectively, our results highlight for the first time that DEP-1 can promote the pro-invasive cell functions of highly invasive basal-like breast cancer cell lines.

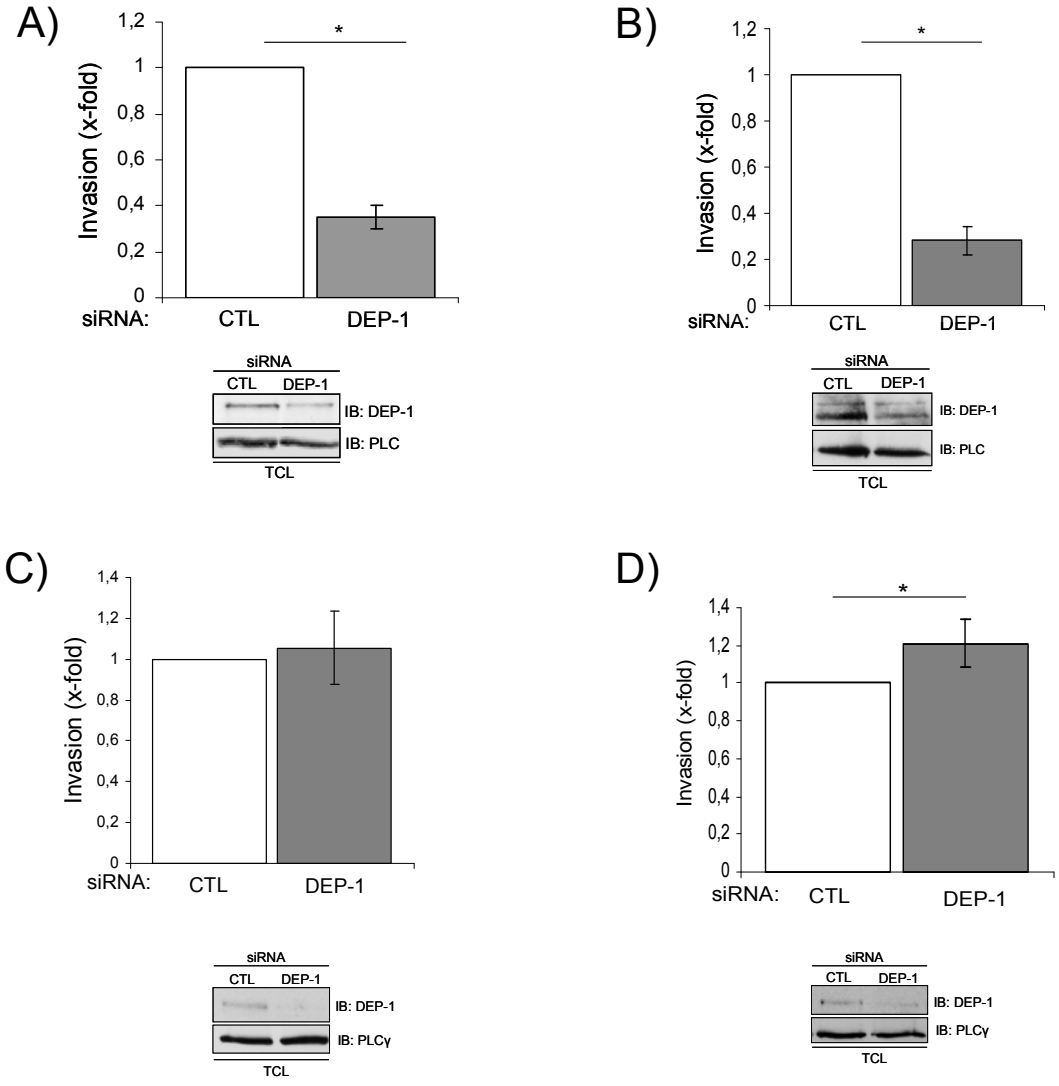


Figure 5: DEP-1 promotes invasion in basal-like breast cancer cells.

(A) MDA-MB 231 cells transfected with control (CTL) or DEP-1 siRNAs were submitted to Matrigel invasion assay (n=3). Inserts were coated with 3,8 mg/ml Matrigel. 1 x 10^5 cells were seeded in the upper invasion chamber. DMEM-5% FBS was added to the bottom chamber and cells were allowed to invade Matrigel for 20h. **(B)** HS578T cells transfected with control (CTL) or DEP-1 siRNAs were submitted to Matrigel invasion assay (n=3) as in (A). Inserts were coated with 3,7 mg/ml Matrigel. **(C)** T47D and **(D)** SK-BR3 cells were treated as in (A) and plated to inserts coated with 2 mg/ml or 1mg/ml Matrigel, respectively. 10^5 cells were seeded in the upper invasion chamber. DMEM-5% FBS was added to the bottom chamber and cells were allowed to invade Matrigel for 20h. Results shown are means ± SEM

of cells that invaded Matrigel and that are on the lower side of the polycarbonate membrane of the insert. Results are representative for 3 independent experiments. p-value were calculated with Mann-Withney U-test * $p < 0.05$

Overexpression of DEP-1 YY/FF mutant in MDA-MB 231 cells correlates with decreased Src and Cortactin activation and impaired invasion.

Our laboratory previously demonstrated that DEP-1 promotes Src activation and Src-dependent biological functions in endothelial cells in response to VEGF (160, 471). We further showed that DEP-1 phosphorylation on Y1311 and Y1320 is required for the mediation of these events. To demonstrate that DEP-1 indeed regulates biological cell functions via Src activation in breast cancer cells, MDA-MB 231 cells were transiently transfected with a moderate quantity of empty vector (pmT2), DEP-1 WT and DEP-1 Y1311F/Y1320F (YY/FF), which was previously shown to block Src activation (471). Figure 6A shows that following FBS stimulation, phosphorylation of Src and Cortactin is impaired in cells expressing the DEP-1 YY/FF mutant compared to cells transfected with empty vector of expressing WT DEP-1. Thus, DEP-1 mediates Src activation and the induction of pro-invasive signalling via tyrosine 1311 and 1320 in MDA-MB 231 cells. The capacity of MDA-MB 231 cells overexpressing a subtle quantity of empty vector (pmT2), DEP-1 WT or DEP-1 YY/FF mutant to invade Matrigel was also tested (Fig. 6B). Similarly, we found that the expression of DEP-1 YY/FF decreased the invasion of MDA-MB 231 compared to cells expressing pmT2 or WT DEP-1. Our results therefore demonstrate that subtle overexpression of WT DEP-1 leads to the increased activation of Src, Src-dependent pro-invasive signalling and mediates invasion in MDA-MB 231 cells. They also show that Y1311 and Y1320 of DEP-1, which are required for the promotion of Src activation, are involved in the induction of Src-dependent functions in MDA-MB 231. These results thus support the conclusion that DEP-1 contributes to the invasive phenotype of MDA-MB 231 cells through the activation of the Src pathway.

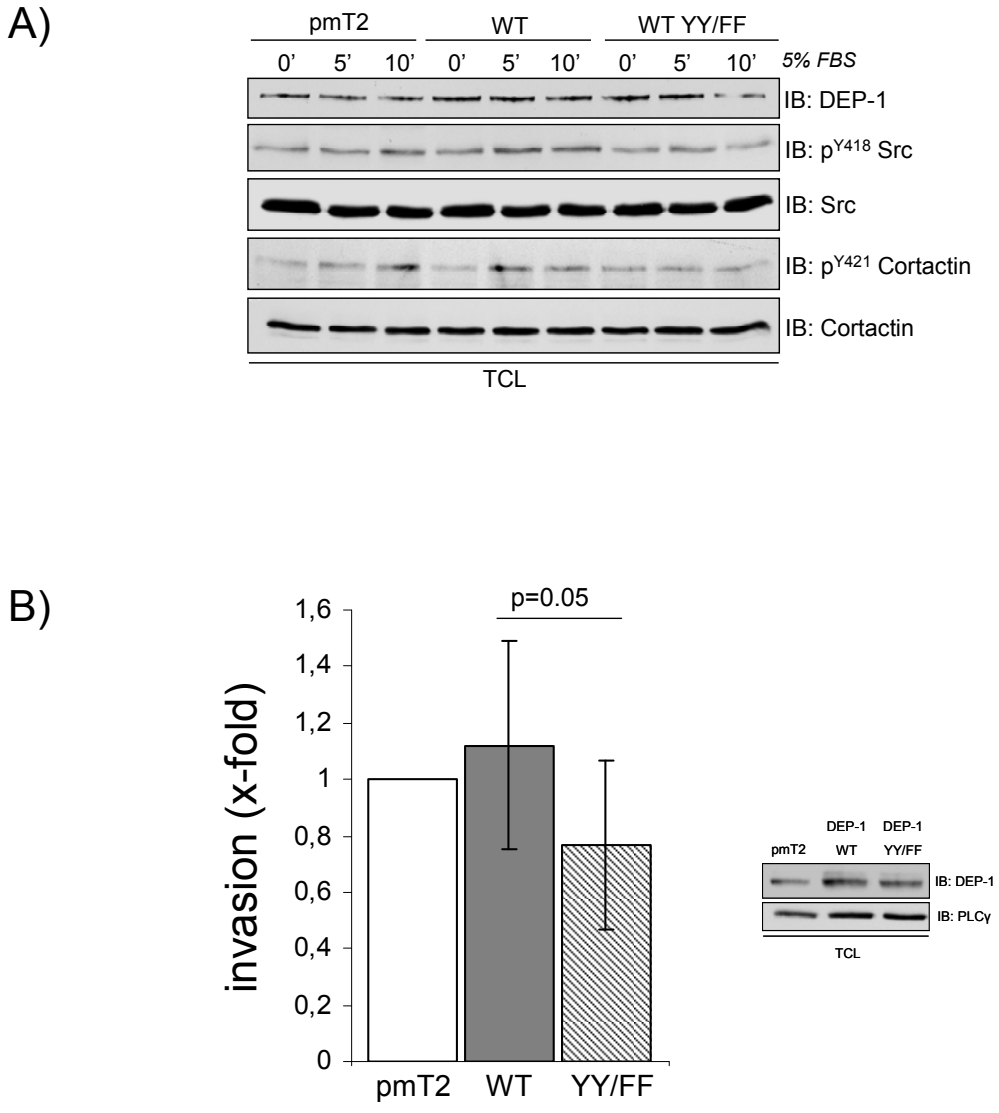


Figure 6: Overexpression of DEP-1 YY/FF mutant in MDA-MB 231 cells correlates with decreased Src and Cortactin activation and impaired invasion.

(A) MDA-MB 231 cells were plated at 3 x 10^4 cells/cm² and transfected with empty vector (pmT2), DEP-1 WT or DEP-1 YY/FF mutant. Forty hours post transfection, cells were stimulated with 5% FBS at given time points, lysed and immunoblotted for DEP-1 expression, Src Y418 and Cortactin pY421 phosphorylation. Overexpression of DEP-1 YY/FF mutant inhibited Src Y418 and Cortactin pY421 phosphorylation. (**B)** Cells transfected as in (A) were submitted to Matrigel invasion assay (n=3). 10^5 cells were seeded on a Matrigel coated polycarbonate membrane with a concentration of 3,55 mg/ml. DMEM-5% FBS was added to the bottom chamber and cells were allowed to invade Matrigel for 20h. Cells were then fixed and stained with crystal violet. Cells that invaded Matrigel and were found at the lower polycarbonate membrane were counted and DEP-1 YY/FF overexpressing cells compared to DEP-1 WT overexpressing cells. * $p< 0.05$

DEP-1 expression promotes the pro-invasive phenotype of the metastatic MDA-MB 231 cell variant 1833.

An essential step for cancer progression is the ability of tumour cells to form metastases at distant sites. In breast cancer, the most preferred site of metastases formation is the bone (509, 510). Interestingly, Src activity was reported to be increased in the metastatic explants (variant 1833) of MDA-MB 231, which formed bone metastases in a Src-dependent manner (360). Based on our results obtained in MDA-MB 231 cells correlating DEP-1 expression to the activation of the Src pathway and cell invasion, we were therefore interested in determining if DEP-1 expression was similarly increased in this metastatic cell line. Figure 7A shows that increased phosphorylation of Src Y418 was observed in 1833 cells compared to the parental cells, as previously reported. Importantly, higher expression of DEP-1 as well as activation of Cortactin and FAK were also detected in the 1833 metastatic variant, consistent with its increased ability to invade Matrigel compared to the parental MDA-MB 231 cells (Fig. 7B). To determine the implication of DEP-1 in the invasive capacity of 1833 cells, MDA-MB 231 and 1833 cells were transfected with control or DEP-1 siRNAs. Figure 7C shows that DEP-1 silencing led to decreased invasion of both the parental MDA-MB 231 and 1833 cell lines compared to controls, although the reduction was more pronounced in the parental cells (Fig. 7C). Thus, in

the bone metastatic MDA-MB 231 subpopulation 1833, the higher amount of DEP-1 expression induces a stronger Src-dependent pro-invasive signalling leading consequently to more invasive 1833 cells compared to the parental MDA-MB 231 cells.

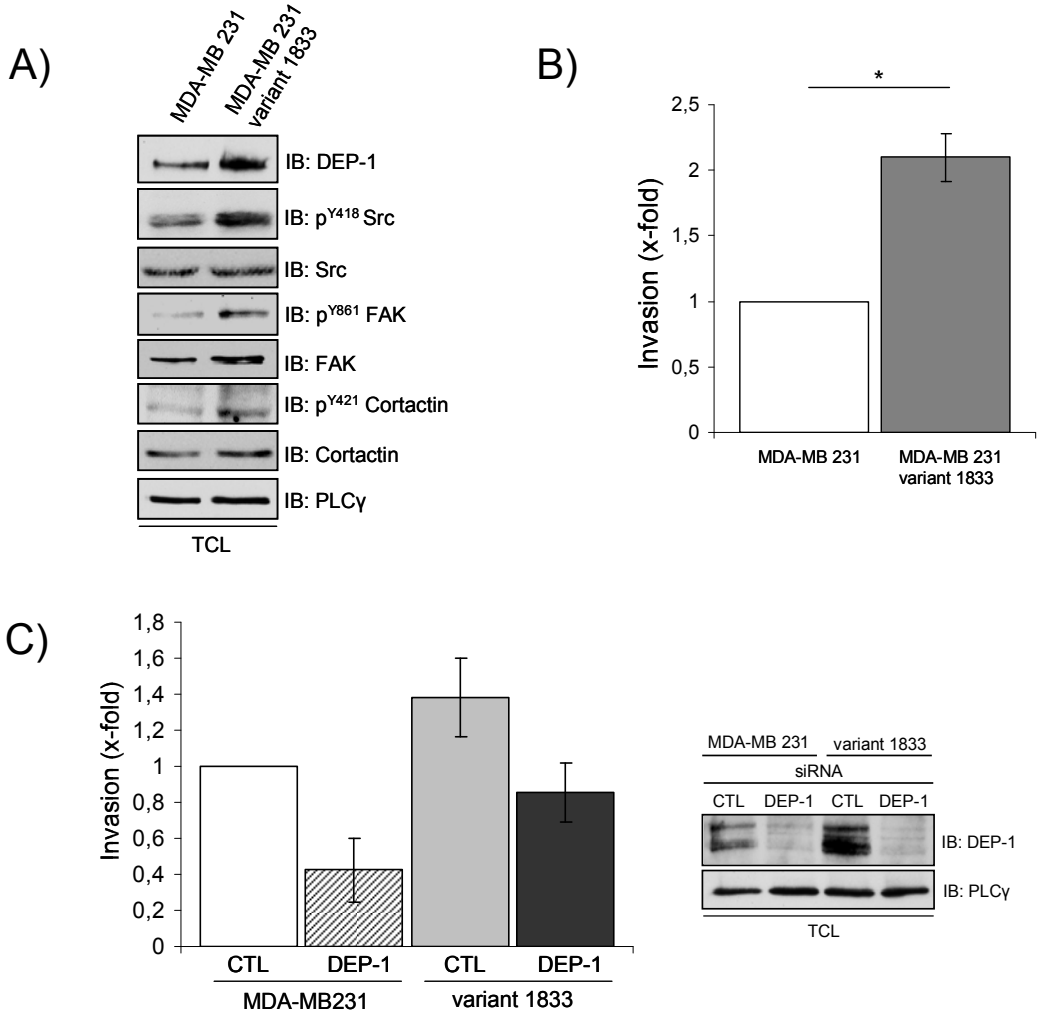


Figure 7: DEP-1 expression promotes the pro-invasive phenotype of the metastatic MDA-MB 231 cell variant 1833.

(A) MDA-MB 231 cells and the bone-metastatic explant 1833 were plated at 2 x 10^4 cells/cm² for 4d and then lysed. DEP-1 expression level and the phosphorylation levels of Src Y418, Cortactin Y421 and Y861 FAK were determined. The enhanced phosphorylation of Src and Cortactin correlates with higher DEP-1 expression in the metastatic explant 1833. **(B)** Cells were submitted to Matrigel invasion assay (n=3). Inserts were coated with 50 µl Matrigel (3,8 mg/ml). 10^5 cells were seeded in the upper invasion chamber. DMEM-5% FBS was added to the bottom chamber and cells were allowed to invade Matrigel for 20h. Cells were fixed and stained with crystal violet. Cells which invaded Matrigel and were located at the lower membrane of the insert were counted. **(C)** MDA-MB 231 and the metastatic explant 1833 were plated at 5 x 10^3 cells/cm^2 and transfected with control (CTL) or DEP-1 siRNA. Forty-two hours post transfection cells were submitted to Matrigel invasion assay (n=3). Inserts were coated with 3.8 mg/ml Matrigel and invasion test was done as described in (B). Results shown are representative for 3-4 independent experiments. * p-value < 0.05, Mann-Withney-U-test.

Expression of DEP-1 in human breast tumours is associated with an anterior breast cancer and might be unfavourable for patient's outcome.

The results presented above suggest that DEP-1 expression is implicated in the promotion of migration and invasion in high invasive breast cancer cell lines. To determine the clinical relevance of our observations, we analyzed DEP-1 expression by immunohistochemistry on a tissue microarray (TMA) encompassing tumour samples from 128 patients. Tumour specimens were evaluated with respect to the presence or absence of DEP-1 expression (Fig. 8A). Analysis of the data revealed that DEP-1 expression was also associated with a previous history of breast cancer in the same or the opposite breast, which was diagnosed more than five years before the actual malignancy (p-value 0,024, Pearson correlation). Moreover, DEP-1 expression seems by trend to be associated with lower overall survival of breast cancer patients (p-value 0.1, Pearson correlation). Further analysis also highlighted that patients with DEP-1 positive tumours showed by a trend reduced overall survival compared to the group with DEP-1 negative tumours (Fig. 8B), suggesting that DEP-1 expression is unfavourable for clinical outcome of the patients. Altogether, our results suggest for

the first time an oncogenic role for DEP- 1 in invasive breast cancer, potentially promoting cancer progression and the formation of metastases. Accordingly, DEP-1 expression might be unfavourable for the clinical outcome of breast cancer patients and could be a novel therapeutic target in breast cancer.

A)

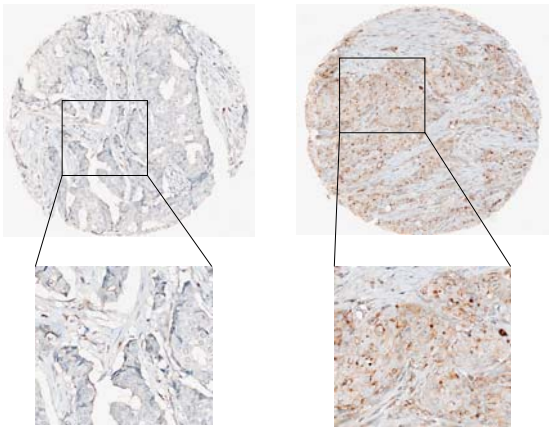


Table1. Pearson correlation analysis in 128 breast cancer patients

prognostic factor	p-value
anterior breast cancer (more than 5 years ago)	0.024

B)

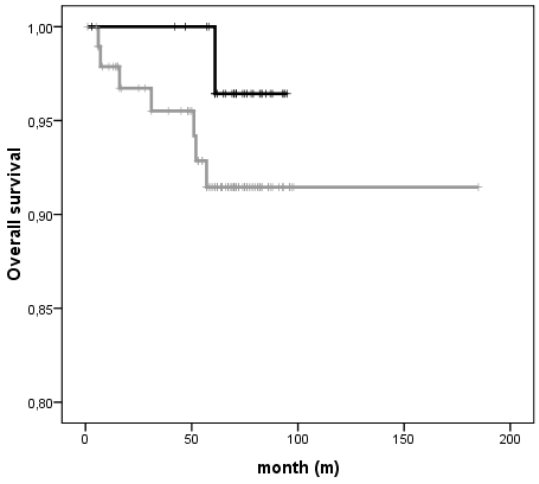
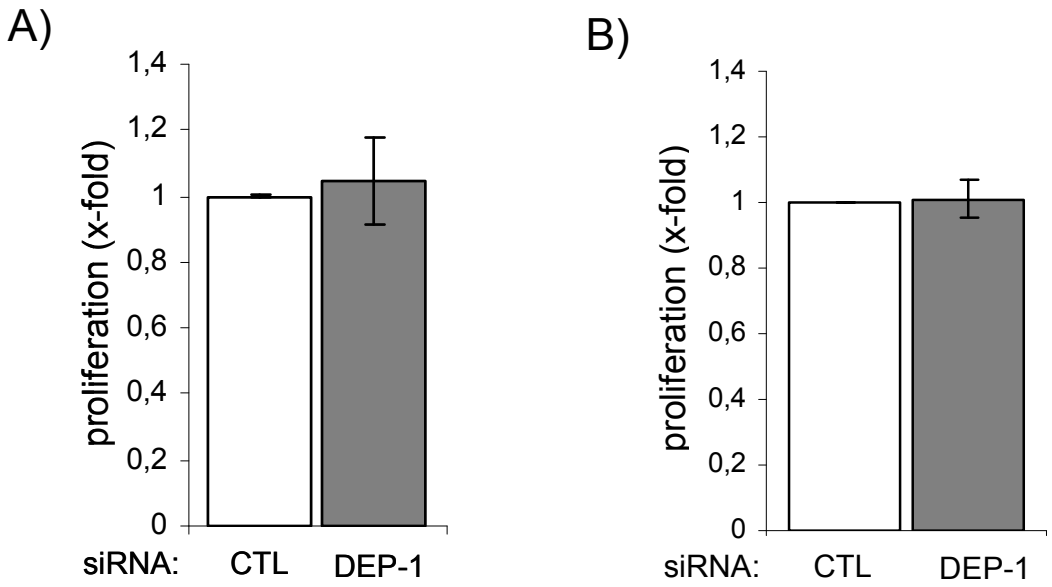


Figure 8: Expression of DEP-1 in human breast tumours is associated with an anterior breast cancer and might be unfavourable for patient's outcome.

(A) Representative cores of a tissue microarray of breast cancer samples illustrate negative and positive DEP-1 staining. Table1 shows the p-values of Pearson/Spearman correlation of an anterior breast cancer history. **(B)** Kaplan-Mayer analysis coupled with a log Rank-test revealed a trend to reduced overall survival of patients with DEP-1 expressing tumours (grey line) versus patients with DEP-1 negative tumours (black line).

Supplemental Figures



Supplemental Figure 1: Cell proliferation in DEP-1-depleted and control MDA-MB 231 and HS578T cells.

(A) MDA-MB 231 cells were plated between 20000 cells/cm^2 and 30000 cells/cm^2 20 h before transfection. Cells were transfected as mentioned in Material and Methods. Twenty-four hours post-transfection cells were trypsinized and counted. Ratio between control and DEP-1-depleted cells was shown as x-fold increase. Results are representative of 9 individual experiments. **(B)** HS578T cells were plated between 20000 cells/cm^2 20 h before transfection with DEP-1 or control siRNA. Twenty-four hours post transfection cells were trypsinized and counted. Ratio between control and DEP-1-depleted cells was shown as x-fold increase. Results are representative of 3 individual experiments.

Discussion

In this study we demonstrate that DEP-1 expression is higher in basal-like breast cancer cell lines compared to the luminal-like cell lines tested. DEP-1 depletion correlates with decreased Src Y418 phosphorylation in basal-like cells. Further analysis of two basal-like breast cancer cell lines (MDA-MB 231 and HS578T) revealed that DEP-1 promotes migration and the proper localisation of Cortactin and Src at the leading edge of migrating cells. We showed further that invasion is decreased when cells express the DEP-1 YY/FF mutant suggesting that DEP-1 tyrosines are impliqued in Src activation in MDA-MB 231 cells. Importantly, DEP-1 expression levels are increased along with elevated Src, Cortactin and FAK activity in the metastatic MDA-MB 231 cell variant 1833, suggesting that DEP-1 is implicated in the metastatic process of MDA-MB 231 cells. Notably, clinical data indicate that DEP-1 expression correlates with an anterior breast cancer and seems to be by trend associated with reduced overall survival of breast cancer patients, confirming our in vitro results and signifying that DEP-1 expression may be a marker to predict the risk to develop metastases.

In this work we showed that DEP-1 expression is elevated in highly invasive basal-like breast cancer cells compared to luminal-like cell lines. These results were unexpected since DEP-1 is reported to behave as a tumor suppressor (392, 394, 399). However, despite the fact that several studies reported that DEP-1 expression inhibits the proliferation of different cancer cell types including breast cancer cells (213, 392, 402), our results nonetheless suggest the novel idea that DEP-1 could also promote tumor progression. In agreement with this hypothesis, we demonstrated that DEP-1 mediates the migration and invasion of breast cancer cells via its ability to activate Src, a well-characterized promoter of cancer cell migration, invasion and metastases formation (493-495, 511). Moreover, it was shown that Src preferentially promotes

the establishment and survival of latent breast cancer metastases to the bone, favoring the activation of the CXCL12-CXCR4-AKT pathway and the resistance to TRAIL-induced apoptosis (360). Interestingly, we also previously revealed a role for DEP-1 in endothelial cell survival, via the activation of a Src-Gab1-AKT pathway (160). As supplementary evidence, our results also show that a metastatic explant of MDA-MB 231 cells express higher DEP-1 levels compared to the parental MDA-MB 231 cell line, and that this correlates with higher phosphorylation of Src on Y418 as well as higher invasive capacity. In light of all of these observations, our data therefore suggest that elevated DEP-1 expression in breast cancer cells could promote cancer progression and the formation of metastases.

To further support this hypothesis and investigate the clinical relevance of our observations, we performed IHC analysis of DEP-1 expression in 128 tumor samples of human breast cancers. Interestingly, as could have been expected based on its known tumor suppressive functions, we did not find an association between decreased DEP-1 expression and advanced breast cancer stages. In contrast, we found that its expression correlates with an anterior breast cancer history and seems to be associated with reduced overall survival of breast cancer patients. The latter results thus support the results from our in vitro studies and suggest that DEP-1 expression could contribute to cancer progression and the metastatic process in breast cancer.

We also showed that DEP-1 is localized at the leading edge of migrating cells, allowing the recruitment and the localisation of Src, Cortactin and Actin to these sites. Src-dependent activation of Cortactin maintains the Actin assembly at the leading edge, promoting the formation of protrusions and lamellipodia (499, 500). Thus, DEP-1 localisation at the leading edge very likely promotes local Src activation and cytoskeletal organization required for the formation of lamellipodia (Fig. 4). In addition, Cortactin has also been reported to regulate membrane trafficking and the secretion of ECM-degrading proteins through the delivery of MMPs and proteases at the cell-substratum interface (498, 512). Consistent with these functions, and similarly to what was reported for Src, elevated expression of Cortactin was shown to

increase the number of bone metastases (360, 507). Based on these results, it can be speculated that a DEP-1-Src-Cortactin signalling pathway initiates organized and directed cell movement leading to cancer cell invasion and the formation of distant metastases (499, 500). Likewise, DEP-1 also increased the phosphorylation of FAK in basal-like breast cancer cells, which is another Src substrate involved in the promotion of cell migration and invasion that is overexpressed and amplified in breast cancer (513, 514). FAK is phosphorylated on several residues including tyrosines and serine/threonines. Phosphorylation on these residues can be induced by several stimuli including growth factors as VEGF and PDGF as well as phorbol ester (515-517). FAK possesses six tyrosine and four serine residues located in different domains. When FAK is inactive, its conformation is closed. Following integrin-mediated binding to ECM the FERM domain will be replaced and allow the autoposphorylation at Y397 and the binding of SH2-domain including kinases such as Src to this residue. Y397 is located adjacent to the kinase domain of FAK and is the major tyrosine phosphorylation site. Src binding to Y397 via its SH2 domain induces Src activation which leads to the phosphorylation of additional FAK residues such as Y576, Y577 and Y861 resulting in full activation of FAK (121, 518-521). Y925 was also identified as residue phosphorylated by Src and is associated with E-cadherin deregulation and integrin adhesion dynamics in EMT (494, 522).

Altogether, these results therefore support the correlation made between higher invasiveness and aggressiveness of basal-like breast cancer cells in relation with the expression of DEP-1, and that its ability to regulate the Src pathway may in part be responsible for these characteristics.

The apparent discrepancy between our observations and those of previous studies that highlighted the tumor suppressor role of DEP-1 suggests that during cancer progression, the tumour suppressive functions of DEP-1 must be reduced or inactivated. Consistent with this idea, it is important to consider that LOH (loss of heterozygosity) of the PTPRJ gene leads to reduced amounts of PTP activity in the cells, which we have shown are appropriate for the optimal activation of Src (471). In

addition, a number of mutations in the extracellular and intracellular domain of DEP-1 have been identified in human cancers, and these could also contribute to modifying the growth suppressive functions of DEP-1 (395, 399, 400, 403). Indeed, a missense mutation in the KIM domain of DEP-1 (K1017N) was found in primary breast tumors, but most enriched in brain metastases, suggesting that this mutation of DEP-1 is advantageous to cancer progression (403). The KIM domain (aa 1013-1024) allows DEP-1 to associates with and dephosphorylate ERK1/2, which are major activators of proliferation (149, 275, 404). Thus, the K1017N DEP-1 variant found in breast cancer metastases might be inactivated with respect to its ability to dephosphoyrlate ERKs and inhibit proliferation, but still able to activate the pro-invasive Src pathway. In addition, as PTPs are regulated by oxidation and that this is increased in most cancer cells, it is also conceivable that oxidation might contribute to the attenuation of PTP activity to reduce the ability of DEP-1 to inactivate growth promoting pathways, but still be sufficient to allow the dephosphorylation and activation of Src. Consistent with this idea, FLT3-ITD-induced cell transformation in AML was recently shown to depend on high Src activation and the oxidative inactivation of DEP-1 (523). Thus, alterations of DEP-1 activity via the presence of inactivating mutations or via increased oxidation could represent an advantage for tumor cells, allowing them to respond to the selective pressure towards Src activation and metastasis formation.

Interestingly, other PTPs that can activate Src are also believed to display oncogenic potential. PTPε, PTP1B and PTPα are examples of PTPs which can activate Src and potentially promote cancer progression (162, 315, 406, 426, 427). However, results of PTPα in cancer are in part controversial. For instance, PTPα expression was shown to correlate with a low tumour grade and cell growth inhibition, while another study recently demonstrated that PTPα expression promotes Src activation and subsequently anchorage-independent growth and cell survival in ER-negative breast cancer and colon cancer cell lines (340, 407, 408). Consistently, the closely related PTPε was also found to be overexpressed in various transgenic mouse mammary tumor models that are Her2/Neu- or HRas-induced (523).

Moreover, it was recently demonstrated that PTPε expression promotes anchorage-independent growth and breast cancer cell survival, further indicating its cancer-promoting role (411). The results presented herein provide for the first time hints that DEP-1 could be implicated in the transformation process of cancer cells due to the activation of Src, similarly to what was shown for other PTPs. Whether these PTPs have a positive or negative role is dependent on the cellular context and on molecular events that take place during cancer progression such as mutations, increased oxidation and expression levels. Thus, a body of evidence suggest that a number of PTPs have the potential to promote cell transformation and subsequently cancer progression. Although results are in part controversial, the different experimental design of the studies and the probable tumor-promoting role of PTPs during different steps of cancer evolution (initiation versus progression) have to be taken into consideration. Further work has to be done to clarify the role of PTPs in a cancer stage-dependent manner.

In conclusion, we report for the first time that DEP-1 can promote a pro-invasive phenotype in breast cancer cells, and that its expression could be associated with an unfavourable outcome for patients. This identifies DEP-1 as a novel and essential player during breast cancer progression and a predictor for the risk to develop metastases.

Acknowledgements

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CHAPTER V

Discussion

The aim of our work was to characterize the role of DEP-1 in the regulation of VEGF-dependent angiogenic processes. Previous work in our laboratory showed that DEP-1 dephosphorylates VEGFR2 at Y1054/1059, leading to global attenuation of VEGFR2 phosphorylation in confluent endothelial cells. Under these conditions major signalling pathways including ERK1/2 are inhibited while the Src-Gab1-AKT pathway is activated (160). Previously published studies demonstrated that DEP-1 mediates cell contact inhibition through the dephosphorylation of VEGFR2 in the VE-cadherin complex at adherens junctions (149). Our work added an additional aspect to the existing model, as we showed that DEP-1-mediated activation of the Src-Gab1-AKT pathway in adherens junctions induces cell survival. Under these conditions, DEP-1 activates Src via the dephosphorylation of the inhibitory Y529. However, the molecular mechanism implicated in DEP-1-mediated Src activation remained poorly defined. A number of previous studies demonstrated that DEP-1 could be phosphorylated on tyrosine in different cell types following growth factor stimulation or treatment with pervanadate (212, 337). However, phosphorylation sites and their effect on DEP-1 activity and DEP-1-mediated cell function remained unknown. Thus, the aim of our work was to better characterize DEP-1 phosphorylation sites and their impact on DEP-1 activity and function. Based on the positive role of DEP-1 in Src activation and the crucial role of Src in many cellular functions, we also wanted to define the influence of DEP-1 on biological function. Our recent work demonstrates that DEP-1 is transiently phosphorylated on the tyrosine residues Y1311 and Y1320 in its C-terminal tail in response to VEGF. These residues are required to bind the SH2 domain of Src, leading to Src activation through the dephosphorylation of its inhibitory Y529. In addition to the promotion of cell survival, our work demonstrated that DEP-1-dependent Src activation also induces angiogenic cell responses including vascular permeability, capillary tube formation

and endothelial cell invasion. The involvement of phosphorylated Y1311 and Y1320 in Src activation allowed us to propose that DEP-1 can activate Src via the "phospho-displacement" mechanism similar to what was proposed for PTPα. Src binds via its SH2 domain to the phosphorylated Y1311 and Y1320 of DEP-1, inducing conformational changes of Src. The phosphorylated inhibitory Y529 of Src is released from the intramolecular binding to its SH2 domain and is dephoshophorylated by DEP-1, leading to Src activation

Moreover, we identified a threonine residue (T1318) in proximity to Y1320 in DEP-1 that is constitutively phosphorylated and displays a consensus sequence for the serine/threonine kinase CK2. Indeed, DEP-1 T1318 is phosphorylated by CK2 as demonstrated in experiments using the specific CK2 inhibitor (TBCA) or when CK2 was overexpressed in cells. Upon VEGF stimulation, DEP-1 T1318 phosphorylation decreases concomitantly with increased phosphorylation on Y1311 and Y1320. Thus, it can be speculated that the negative charge provided by the phosphorylated threonine residue 1318 promotes the VEGF-dependent phosphorylation of Y1311 and Y1320 in endothelial cells and increases the affinity of Y1320 to bind Src. This hypothesis was supported by a sequence analysis of phospho-mimicking mutations of T1318 (T1318E or T1318D) using Scansite (http://scansite.mit.edu/), a software predicting protein interaction (478). These results thus suggest that the phosphorylation of DEP-1 T1318 exerts a regulatory control over DEP-1 tyrosine phosphorylation. This hypothesis is further supported by the observation that a DEP-1 T1318A mutant showed reduced the tyrosine phosphorylation and impaired ability to bind and activate Src in response to VEGF stimulation. Hence, these results showed for the first time that DEP-1 tyrosine phosphorylation and substrate specificity is regulated by the phosphorylation on a proximal threonine residue.

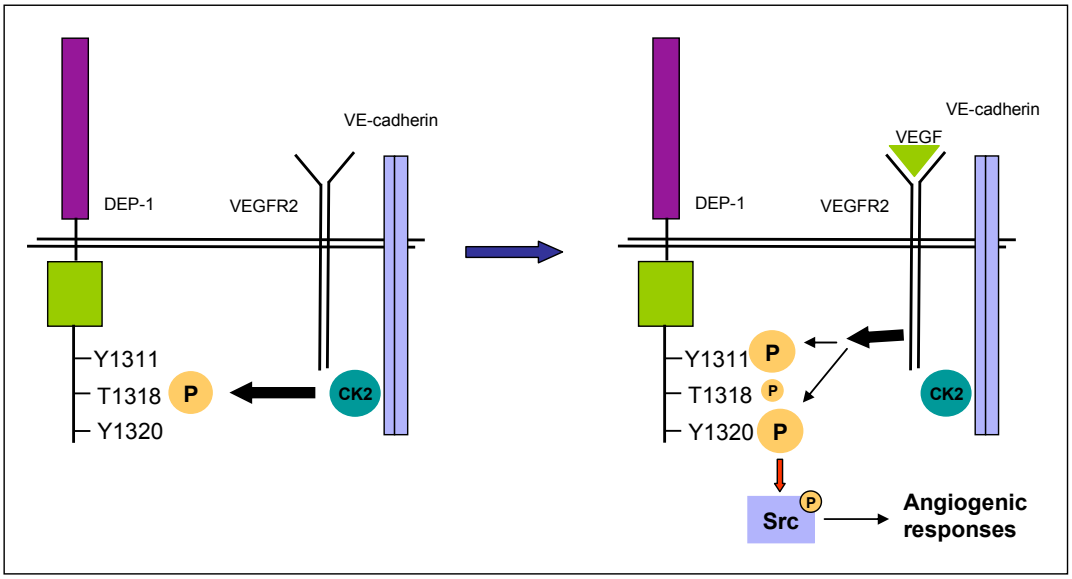

Figure 16: DEP-1 threonine phosphorylation promotes VEGF-induced DEP-1 tyrosine phosphorylation and Src activation.

At basal levels DEP-1 is constitutively phosphorylated on T1318 via CK2. The negative charge of DEP-1 T1318 phosphorylation increases the VEGF-dependent phosphorylation of tyrosine 1311 and 1320 in endothelial cells. DEP-1 tyrosine phosphorylation is required for Src activation and angiogenic responses in endothelial cells.

5.1 Mechanisms to regulate DEP-1 activity and function

The results presented in this work showed that DEP-1 is phosphorylated on its C-terminal tail in endothelial cells. DEP-1 phosphorylation represents a regulatory mechanism to control its substrate specificity towards Src. It can be supposed that supplementary control mechanisms such as oxidation, modulation of its expression levels and ligands exist in cells and modulate the regulation of DEP-1 activity and consequently the dephosphorylation of DEP-1 substrates.

5.1.1 Implication of DEP-1 in the recruitment of signalling molecules

The phosphorylation of DEP-1 Y1311 and Y1320 is induced following VEGF stimulation. DEP-1 tyrosine phosphorylation is required for Src activation, but interestingly, the sequences downstream of Y1311 (YQNT) and Y1320 (YENL) also correspond to the consensus sequence of Grb2 binding (YXNX). Grb2 is a docking protein involved in the recruitment of many proteins such as Gab1 to RTKs at the cell membrane (124, 147, 524, 525). We showed previously that the scaffold protein Gab1 is located at adherens junctions in endothelial cells and is involved in the induction of cell survival and cell motility (124, 160). Moreover, it was suggested that DEP-1 is required for Gab1 recruitment to the VE-cadherin complex. Thus, it might be possible that DEP-1 Y1311 and Y1320 contribute to Grb2-mediated Gab1 recruitment into adherens junctions, inducing Gab1-dependent signalling such as endothelial cell survival.

Further potential phosphorylation sites as well as a PDZ class II binding domain (GYIA) are present in DEP-1, which could have roles in the regulation of DEP-1 activity, DEP-1-mediated signalling and endothelial cell functions. For instance, the DEP-1 PDZ domain could be an additional binding site for Src via syntenin. Syntenin is a scaffolding protein containing tandem PDZ domains and was reported to bind DEP-1 (526, 527). Interestingly, syntenin was shown to bind Src in a human melanoma model. The interaction of syntenin and Src correlates with higher Src/FAK complex formation, the promotion of cell motility and invasion as well as metastasis formation in vivo (528). Thus, in addition to phosphorylated DEP-1 tyrosines, syntenin could also contribute to the binding of Src to DEP-1, promoting Src activation and biological cell functions.

5.1.2 Different DEP-1 expression levels define distinct endothelial cell functions

Notably, DEP-1 is a highly active phosphatase in vitro (175, 318). Our work has revealed that DEP-1 is similar to a number of other Src-activating PTPs in that it has a higher specificity towards the Src inhibitory Y529 than the activatory Y418. Nonetheless, DEP-1 can also dephosphorylate the Src activating tyrosine residue 418 in vitro (318). Consistent with these observations, we demonstrated that DEP-1 specificity towards Y418 and Y529 of Src in HEK293T cells as well as in endothelial cells depends on the DEP-1 expression level. High DEP-1 expression levels in cells decrease DEP-1 specificity, as we observed the dephosphorylation of Y529 and Y418 of Src. Under these conditions, Src activity is decreased in cells. In contrast, moderate DEP-1 expression is optimal for appropriate Src activation and induction of endothelial cell functions. In agreement with our results, CD45 can also dephosphorylate Src Y418 and Y529, leading either to increased or attenuated Src activity in T cells (237, 529). It was suggested that the negative role of CD45 on Src activation could be explained by high levels of CD45 expression or abnormal expression of different CD45 isoforms (462, 530). Based on these data and our results, it can be speculated that a large quantity of DEP-1 in cells decreases Src activation and endothelial cell function including the remodelling of cell-cell junctions and capillary formation, while moderate DEP-1 expression levels stimulate Src activity and its downstream pathways. This hypothesis is consistent with a report that analyzed the in situ expression of DEP-1 by immunohistochemistry in a wide range of tissues and cells. This report shows that DEP-1 is moderately expressed in endothelial cells (531). Moreover, in vivo data showed that DEP-1 expression is downregulated in migrating and proliferating cells during repair of injured vessels, supporting our hypothesis that decreased DEP-1 expression promotes endothelial cell functions (262).

Several lines of evidence gathered in previous studies suggest that DEP-1 expression can increase concomitantly with VE-cadherin expression (149, 191). It is tempting to speculate that VE-cadherins and DEP-1 can have distinct functions in cells depending on their expression levels, which are in part dictated by endothelial cell confluence. Based on these observations, it can be hypothesized that DEP-1 expression in phalanx cells is upregulated concomitantly with VE-cadherin. These cells form non-growing vessels and are tightly aligned and regularly shaped. They are characterized by stable cell-cell junctions achieved by the high expression of VE-cadherin, which tightens the endothelial cell barrier (159). Since high DEP-1 expression attenuates Src activation in endothelial cells, it is tempting to speculate that Src activity is attenuated in quiescent phalanx cells, but that the remaining activity is sufficient to mediate endothelial cell survival via the Src-PI3K-AKT pathway. In contrast to phalanx cells, DEP-1 expression is lower in stalk and tip cells, allowing subsequent Src activation and promotion of permeability, migration and invasion in these cells. Stalk and tip cells mediate the formation of the vascular sprout (10). These cells form the body of the sprout and are responsible for its elongation. Cell-cell junctions are less tight in these cells, probably due to decreased VE-cadherin clustering (532). Thus, we suggest that moderate DEP-1 expression results in optimal Src activation in sprout-forming stalk and tip cells, promoting optimal Src activation and the induction of endothelial cell functions including the loosening of cell-cell junctions, migration and invasion (figure 16). Our hypothesis seems to be supported by preliminary in vivo results in DEP-$1^{-/-}$ mice. Vascular permeability is induced upon VEGF treatment in DEP-1 WT mice, but impaired in DEP-$1^{-/-}$ mice. (unpublished observation, Fournier and Royal).

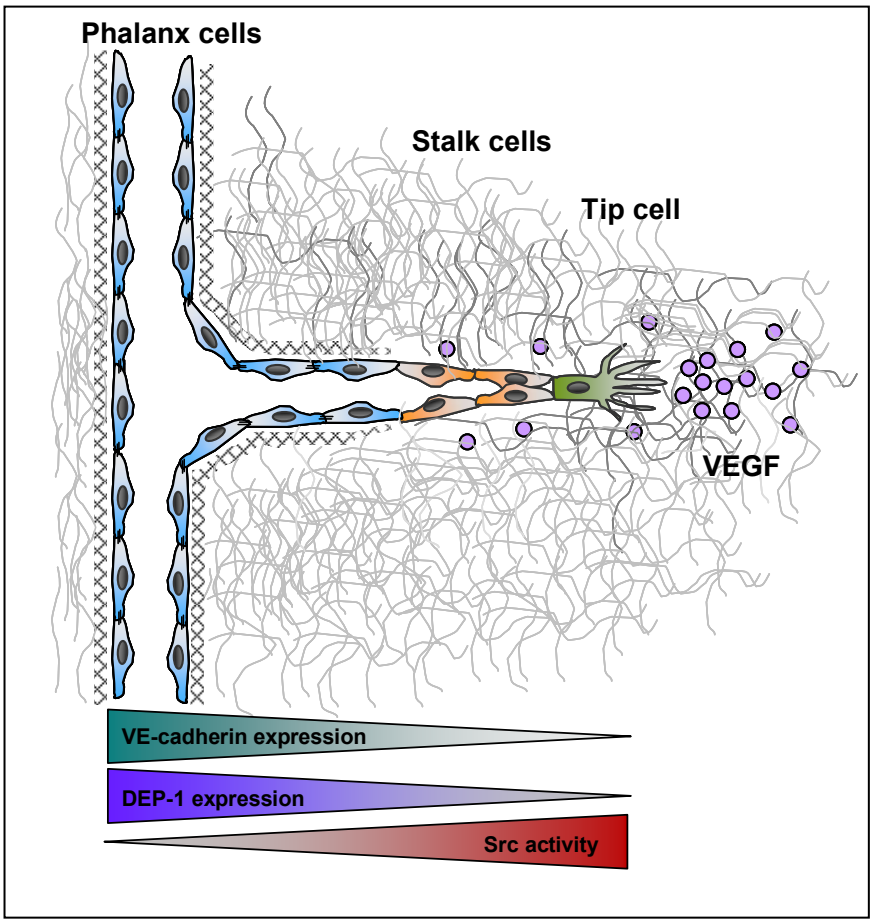

Figure 17: VE-cadherin and DEP-1 expression correlates with activation levels of Src in different endothelial subtypes during angiogenic sprouting.

Quiescent phalanx cells express high levels of VE-cadherins, leading to higher clustering and stable formation of cell-cell junctions. Concomitant DEP-1 expression, which is known to colocalize with VE-cadherin, is increased. This attenuates the level of Src activity due to the partial dephosphorylation of both tyrosines (Y418 and Y529). In sprout-forming stalk and tip cells, the expression level of VE-cadherin and DEP-1 is decreased, resulting in reduced cell-cell adhesion in adherens junctions. Moderate DEP-1 expression levels activate Src to induce endothelial cell functions leading to sprout elongation and the formation of a new vessel. Adapted from (532).

5.1.3 Potential interaction of DEP-1 with the junctional VE-PTP

In addition to DEP-1, other PTPs such as VE-PTP can localize to and regulate the VE-cadherin complex in adherens junctions. Intriguingly, the functional loss of VE-PTP and the knock-in of a DEP-1 mutant with a non-functional intracellular domain during embryonic development result in lethality before embryonic day 10 because of defects in vessel remodelling and abnormal angiogenesis (233, 265). These results provided strong hints that both PTPs have pivotal roles in angiogenesis. Further similarities in structure and function of DEP-1 and VE-PTP can be noticed. For instance, both PTPs can dephosphorylate VEGFR2 to regulate VEGF-dependent signalling and consequently the remodelling of adherens junctions (149, 252, 260, 471). In contrast to DEP-1, VE-PTP associates with VE-cadherin at basal levels and its dissociation from this complex is essential to induce endothelial cell functions in vitro and in vivo (256, 260). Whereas VE-PTP depletion increases VEGF-induced permeability in cells, DEP-1 depletion decreases it, suggesting that both PTPs are implicated in the regulation of the same processes at adherens junction, but interestingly in an opposite manner (260, 471). Moreover, both PTPs can interact with and dephosphorylate junctional proteins such as plakoglobin (260, 274). VE-PTP promotes VE-cadherin-mediated adhesion via plakoglobin, suggesting that VE-PTP is essential to strengthen the junctional barrier (260, 533). It can be speculated

that VE-PTP maintains junctional integrity preferentially in quiescent endothelial cells, resulting in an enhanced endothelial cell barrier. Concomitantly, DEP-1 mediates cell contact inhibition and survival in these cells (149). Thus, DEP-1 and VE-PTP may work together to maintain the quiescent phenotype of phalanx cells in non-growing vessels. Upon activation of endothelial cells, VE-PTP dissociates from the VE-cadherin complex, favouring the loss of the endothelial barrier and subsequent induction of permeability. Moreover, we demonstrated that DEP-1 also promotes vascular permeability via Src activation in VEGF-stimulated endothelial cells. Based on our work, moderate expression of DEP-1 activates Src, and we suggest that this could be the case in stalk and tip cells in growing vessels. Hence, in these cells DEP-1 has a role in promoting permeability and cell motility. It stands to reason that VE-PTP and DEP-1 may cross talk and may regulate each other because they both exert different effects on the endothelial barrier function at adherens junctions. This could be an interesting question to pursue in future studies. For instance, knockdown of VE-PTP could shed light on its role in DEP-1 phosphorylation and activity at adherens junctions. Similarly, phosphorylation and activity of VE-PTP could be elucidated in DEP-1-depleted endothelial cells.

5.1.4 ROS-mediated regulation of DEP-1 and DEP-1-dependent signalling

Reactive oxygen species (ROS) are implicated in the regulation of various signalling pathways downstream of growth factor receptors. Moreover, the thiol in the side chain of cysteines is very sensitive to oxidation, therefore PTPs with their reactive cysteine are pivotal candidates for transmission of ROS signalling (534). In agreement with this, it was reported that RPTPs are prone to oxidation, leading to catalytic inhibition (170). This time-limited reversible inhibition of RPTPs is believed to enhance RTK signalling and to promote cell signal transmission. Considering these

data and the observation that DEP-1 is highly active and a substrate of itself, oxidation of the DEP-1 catalytic cysteine would allow phosphorylation of DEP-1 tyrosine Y1311 and Y1320 in its C-terminal tail, in turn promoting Src binding and subsequent Src activation in response to VEGF. Cells are commonly treated with NAC (N-acetyl-cysteine), a scavenger that can directly interact with ROS, to inactivate ROS-dependent signalling and provide cytoprotection against intracellular ROS damage (535). Consistent with our hypothesis, ROS inactivation by NAC treatment increases PTP activity and subsequently reduces PDGFR signalling as shown in vascular smooth muscle cells (536). This is further supported by our observation that NAC treatment decreased DEP-1 phosphorylation on its C-terminal Y1311 and Y1320 in endothelial cells. These results suggest that DEP-1 or possibly other PTPs such as VE-PTP are more active and dephosphorylate the DEP-1 Y1311 and Y1320 (Spring and Royal, unpublished observations). These preliminary data support our hypothesis that ROS-induced inactivation of DEP-1 increases its phosphorylation of tyrosine 1311 and 1320 in the C-terminal tail. A recent report that ROS-dependent signalling occurs in VEGF-stimulated endothelial cells inducing cell migration and proliferation through the formation of Cys-OH in cysteine-containing proteins provides additional evidence in support of our hypothesis (484, 485). It is of note that CK2 can also be a subject of oxidation. It was demonstrated that ROS application leads to CK2 inactivation in a mouse model of cardiac hypertrophy (483). Thus, it can be speculated that ROS-mediated CK2 inhibition upon VEGF treatment would result in reduced DEP-1 T1318 phosphorylation. Since ROS decrease also PTP activity, the initially reduced PTP activity would allow concomitantly the accumulation of DEP-1 tyrosine phosphorylation in endothelial cells and consequently Src activation. Therefore, the observation that DEP-1 T1318 phosphorylation is reduced after VEGF stimulation could be indicative of the inhibition of CK2 due to ROS production. Based on these data, the regulation of DEP-1 by ROS in response to VEGF is very likely.

Oxidation is also known to occur in cancer (537). Accordingly, it can be speculated that DEP-1 can also be regulated by oxidation in cancer. Recently, a study showed that oxidation is implicated in the transformation process in cells expressing the AML-related FLT3-ITD variant. The same study showed that oxidative attenuation of DEP-1 activity by extensive ROS production contributes to cell transformation in FLT3-ITD variant expressing AML cells (223). At the same time, another group demonstrated that Src activity mediates the transformation process in FLT3-ITD induced AML (538). Since we showed that DEP-1 has a pivotal role in Src activation in endothelial and breast cancer cells, it can be presumed that DEP-1 also activates Src in FLT3-ITD induced AML, promoting cell transformation. These results showed that ROS are implicated in the regulation of DEP-1 in cancer cells, providing further support to our hypothesis that ROS modulate DEP-1 phosphorylation and activity.

DEP-1 can form homodimers following H_2O_2 treatment via the formation of disulphide bonds (184). It can be hypothesized that ROS-dependent formation of DEP-1 higher order complexes could contribute to the rapid and transient attenuation of DEP-1 activity in endothelial cells following VEGF treatment. Therefore, these complexes could be part of a mechanism regulating DEP-1 activity, allowing the phosphorylation of its C-terminal tail and Src activation early after VEGF stimulation. Following oxidation, cysteines in the catalytic domain of DEP-1 could form disulphide bonds leading to dimerization similar to what was shown for PTPα (539). H_2O_2 treatment of PTPα induced rapid and transient formation of disulphide bonds between the catalytic cysteines of each monomer, leading to reversible PTPα inactivation (540). Thus, this may suggest that under oxidative conditions DEP-1 can also form rapid and transient inactive homodimers via oxidized cysteines in its catalytic domain. This hypothesis seems to be in contrast with reports showing that dimerization induced by ligands or by a bivalent antibody results in the formation of an active DEP-1 dimer (191, 198, 199). However, these results were obtained under non-oxidative conditions. It is unlikely that DEP-1 dimerizes via the formation of

disulphide bonds of cysteines in its catalytic domain under non-oxidative conditions. Thus, this may indicate that DEP-1 dimerization due to ligand-induced clustering maintains its catalytic activity under these conditions. This hypothesis may explain the formation of active dimers after treatment with a bivalent antibody (191). Further studies are needed to elucidate the implication of DEP-1 homodimer formation in the cellular response to growth factor stimulation and the implication of an oxidative environment in this process. For instance, a modified cysteinyl-labeling assay was described for detection of reversibly oxidized PTPs in vivo, and this could be used to analyse DEP-1 oxidation and dimerization in response to VEGF (541). Further, specific antibodies against terminally oxidized catalytic cysteines could be used to monitor DEP-1 oxidation in cells as it was already done for other PTPs (542). Mass spectrometry-based analysis could differentiate between oxidized and reduced cysteines of DEP-1 since this method was already used to monitor differences in relative oxidation of different PTPs in tumour cells (543, 544). To determine if DEP-1 forms dimers under oxidative conditions, differentially tagged DEP-1 constructs (i.e. HA- and Flag-tag) could be immunoprecipitated from lysates of untreated and H_2O_2 treated cells. Immunoprecipitates could be analyzed by SDS-PAGE for the formation of higher complexes. This strategy was already used to elucidate the dimerization of other PTPs including PTPα and PTPμ (176, 545).

5.1.5 Regulatory potential of the DEP-1 extracellular domain on DEP-1 activity and function – Implication of DEP-1 ligands

DEP-1 activity and function could also be regulated through specific ligands that bind to its extracellular domain. DEP-1 ligands have remained unknown for a long time. The identification of a component of the extracellular matrix provided initial evidence for a DEP-1 ligand, and recently two specific DEP-1 ligands were identified (546). Syndecan 2 was the first specific DEP-1 ligand to be identified and is

implicated in the regulation of angiogenic processes such as capillary formation (198, 547). Inhibition of syndecan 2 leads to decreased formation of capillaries on Matrigel, similar to what we observed for DEP-1. Moreover, cell spreading and adhesion are impaired in these cells, suggesting a promoting role on angiogenic processes. Interestingly, syndecan 2 was reported to promote cell migration and invasion in different cancer cell types, suggesting a tumour-promoting role (548-550). Thus, it can be speculated that syndecan 2 mediates its pro-angiogenic and tumour-promoting roles in part via DEP-1.

The identification of TSP-1 as a DEP-1 ligand offered new insights into the biology of physiological and tumour-associated angiogenesis. TSP-1 is the first endogenous angiogenic inhibitor discovered (55, 56, 199). It is a matricellular protein and well-known anti-angiogenic regulator on endothelial cells growth, sprouting and motility in vitro and in vivo (55, 56, 551-554). For instance, TSP-1 can inhibit endothelial cell proliferation and neovascularisation under different conditions in vivo (56, 555). Intriguingly, some studies provided evidence that TSP-1 can also have pro-angiogenic roles under specific conditions (556). For instance, it was associated with the increased phosphorylation of junctional proteins including p120 catenin and plakoglobin, subsequently resulting in the induction of permeability (557, 558). Furthermore, TSP-1 can reorganize the cytoskeleton and can induce cell motility in VSMCs and cell spreading in endothelial cells (559-561). Altogether, these studies demonstrate that TSP-1 can promote angiogenesis in specific conditions, despite its mainly negative regulatory roles on angiogenesis. In concordance with these studies, TSP-1 was also reported to have controversial roles in the regulation of neovascularization in cancer (562-566). DEP-1 also has crucial roles at adherens junctions, promoting the phosphorylation of junctional proteins and the induction of permeability similar to what was proposed for TSP-1 (471). Thus, intriguingly, obvious parallels between the double-edged functions of DEP-1 and its ligand TSP-1 occur in angiogenesis. These different and sometimes opposing functions may be dependent on the cellular context and the stimulatory factors involved.

Several studies also provide evidence that TSP-1 possesses anti-tumorigenic properties. For instance, a study revealed that a fragment of TSP-1 is encoded by a tumour suppressor gene. However, the overall involvement of TSP-1 in cancer remains complex and controversial since activatory roles for TSP-1 in tumorigenesis were reported in addition to inhibitory functions (567, 568). Its function as a tumour suppressor inhibiting tumour cell growth is well documented in primary tumours (569-571). Some studies also showed that despite tumour growth inhibition in mammary tumours, TSP-1 promotes metastasis formation to the lung in vivo (572). Interestingly, TSP-1 expression was reported to be low in benign breast lesions but high in breast cancer cells, suggesting that TSP-1 maybe have positive roles cancer promotion (573-575). Additionally, TSP-1 was shown to increase the general capacity of breast cancer cells to invade a matrix in vitro (572, 576). Consistent with these observations, TSP-1 expression was associated with a higher invasiveness in advanced prostate cancer and in pancreatic carcinoma (577, 578). In agreement with this, TSP-1 levels are associated with areas characterized by high intratumoral vessel density, suggesting a promoting role on angiogenesis and in breast cancer (579).

Thus, functionally distinct roles of TSP-1 occur in cancer. TSP-1 expression by the tumour surrounding stroma cells inhibits tumour growth during early stages of cancer. Moreover, TSP-1 could either promote or inhibit tumour neovascularization depending on specific conditions and on the levels of available TSP-1. Byrne and colleagues demonstrated a direct correlation between TSP-1 plasma levels and the breast cancer stages, with highest levels of TSP-1 in metastatic breast cancer (579). During tumour progression, the tumour becomes hypoxic and pro-angiogenic factors such as VEGF are induced, which overrides the inhibitory effect of TSP-1 and results in the angiogenic switch. The overwhelming VEGF secretion counterbalances the negative role of TSP-1 on angiogenesis and results in new vessel formation in the tumour. Concomitantly, high TSP-1 expression increases invasiveness in tumour cells, promoting metastasis formation due to induction of matrix degrading enzymes such as plasmin and the altered regulation of cell adhesion (576, 580-583).

Similarly to the dual role of TSP-1 depending on the cell context in cancer, DEP-1 also displays distinct roles in cancer. Various studies demonstrated a tumour suppressive role for DEP-1 in different cancers. For instance, Keane and colleagues proposed that DEP-1 expression mediates growth inhibition in MCF-7 cells (392). Moreover, the same inhibitory effect of DEP-1 on tumour cell proliferation was shown in the context of colon, thyroid and pancreatic cancer (394, 395, 402, 584). In contrast to these data, our work rather suggests a positive role for DEP-1 in terms of promotion of migration and invasion in a particular subtype of breast cancer cells. The parallels between distinct TSP-1 and DEP-1 functions in cancer are intriguing. It can be speculated that the functional differences of both proteins could be correlated. They may be caused by an altered dose-dependent binding capacity of DEP-1 and TSP-1 due to different expression levels of the proteins or specific mutations in the extracellular domain of DEP-1 in cancer. Furthermore, the availability of TSP-1 as a ligand for DEP-1 can be altered in different stages of cancer since TSP-1 is expressed by tumour surrounding stromal cells in the early stages, whereas tumour cells are the main source of TSP-1 in later stages. Further work will be necessary to clarify this point.

5.1.6 Regulatory potential of the DEP-1 extracellular domain on DEP-1 activity and function – N-glycosylation

N-linked glycosylation is a fundamental post-translational modification and a number of potential N-linked glycosylation sites are predicted in the extracellular and transmembrane domain of DEP-1. Moreover, some sites are identified by glycoproteomic analysis or mass spectrometry in the human plasma or in the liver, and digestion of DEP-1 immunoprecipitates with N-glycosidase revealed that DEP-1 is highly modified by N-glycosylation (263, 585, 586). N-glycosylation regulates protein localization at the cell surface and is implicated in a multitude of cellular

processes including cell-cell or cell-matrix interactions as well as receptor-ligand interactions (587). Since N-glycans were reported to be implicated in the regulation of endothelial cell proliferation and capillary tube formation, it can be speculated that N-glycosylation may represent a regulatory mechanism for DEP-1 function. Interestingly, such a posttranslational modification was demonstrated for two other RPTP and was suggested to alter PTP functions. For instance, two distinctly glycosylated isoforms of PTPα with different molecular weights were identified and it was presumed that they can be distinguished by their ligand specificity (292). Posttranslational modification by N-glycosylation was also described for PTPε. Intriguingly, the transmembrane form of PTPε occurs differentially N-glycosylated depending on the tissue where PTPε is expressed. Thus, this may indicate that N-glycosylation influences PTPε function in a tissue-specific manner (588). Similar roles of N-glycosylation-mediated modifications could be assumed for DEP-1, influencing its capacity to bind its ligand and subsequently its cellular functions.

5.2 The implication of DEP-1 in cancer progression

In this work we identified a novel role of DEP-1 in breast cancer. In contrast to its known tumour-suppressing function in cancer due to cell contact inhibition and the promotion of cell differentiation, we demonstrated its oncogenic potential in basal-like breast cancer cells based on its ability to activate Src. DEP-1 expression is higher in basal-like breast cancer cells, which are known to be highly invasive and more aggressive than luminal breast cancer cell lines. Interestingly, tyrosine 1311 and 1320 of DEP-1, which we previously identified as important residues for Src activation in endothelial cells, are also important for Src activation in breast cancer cell lines. We showed that Src-dependent activation of pro-invasive signalling is impaired in DEP-1-depleted basal-like breast cancer cell lines, consistent with reduced recruitment of Src and Cortactin to the leading edge of these cells. Consequently, cell migration and

invasion are reduced, suggesting that DEP-1 has a pivotal role in cytoskeleton organization and cell motility in highly invasive breast cancer cells. These results are reminiscent of data obtained in macrophages, where DEP-1 was reported to mediate cytoskeletal rearrangements. LPS and CSF-1 treatment induce the formation of membrane ruffles, and DEP-1 is redistributed to these structures (508, 589, 590). Moreover, DEP-1 promotes macrophage spreading, migration and chemotaxis, but does not alter cell proliferation and cell survival. These results indicate that DEP-1 activates important cell functions associated with cytoskeletal rearrangement in macrophages (508, 591).

The clinical significance of DEP-1 expression in breast cancer was addressed by analyzing DEP-1 expression levels in a tissue microarray (TMA) derived from human breast tumours. Consistent with our results showing the involvement of DEP-1 in the promotion of an invasive response, DEP-1 expression is negatively correlated with the overall survival of breast cancer patients. Moreover, DEP-1 expression is positively correlated with an increased probability of a previous breast tumour in the same or the opposed breast. These results suggest DEP-1 expression correlates with a poorer clinical outcome in breast cancer patients. Altogether, we provide initial evidence for the oncogenic potential of DEP-1 in breast cancer.

Several PTPs display oncogenic capabilities. In some cases the oncogenic potential of PTPs is linked to their ability to activate Src as it was proposed for PTPα and PTPε (162, 303, 406). Recently, a study revealed a positive role for PTPε in EGFR-induced cell survival and anchorage-independent growth in breast cancer, providing evidence for the oncogenic potential of PTPε in breast cancer (411). Breast cancer is classified into different subtypes depending on distinct molecular signatures (361-363). According to this molecular taxonomy, some breast cancer subtypes including basal-like and Her2 are associated with an unfavourable prognosis for patients (362). Cancer cells pass through adaptive and selective mechanisms during tumour progression that are accompanied by the appearance of epithelial-to-mesenchymal-transition (EMT) in human solid tumours such as breast cancer. EMT is believed to

be a transient mechanism defining cellular plasticity and is generally associated with higher aggressiveness in breast cancer. The neoplastic progression during cancer development induces the loss of cell polarity and epithelial cell characteristics including the downregulation of E-cadherin and cytokeratins and the gain of mesenchymal markers such as Vimentin and N-cadherin (592, 593). The loss of E-cadherin in carcinomas induces the disruption of cell-cell junctions and promotes EMT. Phosphorylation of proteins associated with E-cadherin such as β-catenin further promotes the loss of cell-cell contacts (594-596). Since DEP-1 is implicated in the regulation of cell-cell junctions, it can be hypothesized DEP-1 could promote EMT. The molecular changes during EMT are accompanied by a more invasive behaviour. Indeed, EMT can play essential roles in cancer cell invasion, when transformed cells start to disassemble from the cell complex and invade tissue surrounding the primary tumour to spread to distant sites. Thus, it is not surprising that EMT is found at the invasive front of cancer cells and is implicated in the formation of metastases (592, 597). A body of literature provides evidence that EMT occurs especially in basal-like breast cancer, which displays high aggressiveness and metastatic spreading (363, 598, 599). It is known that Src and FAK are important mediators of EMT (339, 354, 600). Interestingly, we observed that DEP-1 can regulate Src and FAK activity in basal-like breast cancer cell lines, further suggesting that DEP-1 could be implicated in the mediation of EMT in this breast cancer subtype. Moreover, DEP-1 could have a pivotal role during the disassembly of potentially invasive cells from the cell complex. It is known that activated FAK and Src induce cytoskeleton rearrangements resulting in polarized cells and directed migration (121, 122, 355). Polarized cells form leading edges that are mediated by various proteins including the Src substrate Cortactin. Cortactin is essential to maintain and stabilize lamellipodia and the actin assembly at the leading edge (499, 500). Based on our results that DEP-1-depleted basal-like breast cancer cells fail to recruit Cortactin and Src to the leading edge, it can be suggested that DEP-1 is a positive regulator of cytoskeletal arrangement mediating, their appropriate

localization in migrating cells. Considering these data and our observation that DEP-1 expression is enhanced in basal-like cell lines, it can be hypothesized that DEP-1 could be implicated in the process of EMT, leading to higher cell motility in some breast cancer cells. Thus, it is tempting to speculate that DEP-1 could promote the formation of metastases in basal-like breast cancer.

Interestingly, various studies linked the EMT-like phenotype to tumour propagating cells (TPCs) (601-603). Of note, TPCs were identified in a number of cancer types including that of the breast. They are highly invasive and resistant to chemotherapy, and by definition they can induce de novo tumour growth (604, 605). However, it was suggested that the EMT and TPC cell programs can overlap in some cells, since invading TPC had to undergo EMT to adopt these invasive features (606). Recent studies suggest that TPCs arise from a fully differentiated cancer cells through mechanisms such as EMT that counteract cell differentiation (607-609). Since DEP-1 regulates breast cancer cell invasion and could be implicated in EMT, it could be hypothesized that DEP-1 can also influence TPCs through the promotion of EMT, which is essential to allow cells to invade and migrate. Additionally, the data obtained in the tissue microarray also suggest that DEP-1 expression could be associated with TPCs, as we observed a correlation of DEP-1 expression and the occurrence of a previous breast tumour in patients before the current breast cancer (Pearson correlation p=0,05). In agreement with this, SHP-2 was recently proposed to be a key factor in TPC regulation due to its activating effect on tumour initiating cells and subsequently on tumour maintenance and progression (610). The role of DEP-1 in EMT and the formation of metastases would be an exciting object of future work. For instance, the impact of DEP-1 depletion on the EMT-like phenotype could be analyzed by the evaluation of epithelial and mesenchymal cell surface markers. Moreover, the expression of TPC markers could be analyzed in DEP-depleted cells (611). In vivo experiments could provide insights into the tumour-seeding capacity of DEP-1-depleted breast cancer cells to highlight the potential implication of DEP-1 in the regulation of TPC.

CHAPTER VI

Conclusion

Angiogenesis is a hallmark of cancer allowing the tumour to grow and to invade surrounding tissue. The identification of VEGF as a key regulator of angiogenesis encouraged development of the first anti-angiogenic therapeutic approach targeting VEGF and its receptor. However, although most anti-angiogenic treatments were partially successful in preclinical studies, they only provide moderate benefits for cancer patients. Tumour evasion due to resistance to anti-angiogenic treatment through the upregulation of other redundant angiogenic factors such as FGF is a current problem of these therapies. Additionally, as shown for other anti-cancer treatments, it was reported in a mouse model that anti-angiogenic treatment could induce local tumour invasion followed by accelerated formation of metastases. To improve anti-angiogenic therapies, we need a wider and deeper understanding of the molecular mechanisms underlying the angiogenic process, especially in pathologies such as cancer. Future approaches of anti-angiogenic treatments could target components downstream of several angiogenic factors in addition to the VEGFR2 pathway to attenuate tumour resistance and evasion. Metastasis formation involves the entrance of tumour cells into the blood circulation to escape from unfavourable conditions due to oxygen and nutrient deprivation at the site of the primary tumour. The understanding of the implicated molecular mechanisms leading to the disassembly of tumour cells from the cell complex may be the cutting edge for successful anti-cancer treatments in the future.

The fundamental aim of this doctoral thesis was to define the role of the protein tyrosine phosphatase DEP-1 in angiogenic processes in endothelial cells and in the pro-invasive phenotype of breast cancer cells. To accomplish this aim, the objectives of our first two studies were to define the molecular mechanism of DEP-1-mediated Src activation and its implication in the mediation of endothelial cell functions. In particular, we were interested in characterizing the role of DEP-1

phosphorylation in the regulation of DEP-1 activity and DEP-1-mediated endothelial cell functions. Our results revealed DEP-1 phosphorylation as a major component regulating DEP-1 activity and in particular DEP-1 substrate specificity towards Src. Moreover, our results demonstrate a supplementary level of Src regulation in response to VEGF through the temporal VEGF-dependent control of DEP-1 tyrosine phosphorylation and subsequent Src activation. These studies also revealed DEP-1 as an important positive regulator of the VEGF-induced angiogenic program in endothelial cells and may pave the way for a specific anti-angiogenic therapy targeting DEP-1 or its phosphorylated residues. Our work is among few studies that used DEP-1 depletion by siRNA to highlight this positive role of DEP-1 on endothelial cell functions. To date, the majority of reports studied DEP-1 in the context of its overexpression and focused on its negative role in angiogenesis and cancer.

In concordance with our data obtained in endothelial cells and using DEP-1 depletion as experimental approach, our third study unravelled a novel unexpected role for DEP-1 in breast cancer. In contrast to its initially recognized role as a tumour suppressor, our work demonstrated that DEP-1 promotes the invasive phenotype of human basal-like breast cancer cell lines. Immunohistochemical studies argue for a direct association between DEP-1 expression and the clinical outcome of breast cancer patients, showing that DEP-1 expression tends to reduce the overall survival of breast cancer patients. Thus, these results highlight an oncogenic role for DEP-1 in basal-like breast cancer cell lines. To our knowledge, this is the first study that provides evidence that DEP-1 can act as tumour promoter under certain conditions. Thus, the work we performed in endothelial and breast tumour cells contributed to the discovery of an unsuspected positive role for DEP-1 in the promotion of the Src pathway in these cell systems, and consequently on biological responses that are intimately linked to the activation status of Src in both cellular systems. It is noteworthy that a similar positive role for DEP-1 through the activation of SFK in

immune cells is also being discovered, identifying DEP-1 as a critical regulator of this family of enzymes and of important biological functions.

In conclusion, this thesis investigated important questions leading to the better understanding of the angiogenic process in physiological and pathological conditions. We further the understanding of the role of DEP-1 in endothelial cells and these new insights may contribute to answer current challenges in anti-angiogenic therapies in cancer. Moreover, our results in breast cancer broadened the horizon of DEP-1 function in cancer due to its unexpected tumour-promoting role. Finally, the quantification of DEP-1 expression might also offer a new marker to predict the risk of developing metastases in breast cancer patients and may improve the treatment of breast cancer.

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